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Shigella flexneri ArcA and Fnr regulate iron acquisition and contribute to plaque formation under anaerobic conditions

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Shigella flexneri ArcA and Fnr regulate iron acquisition and contribute to plaque formation under anaerobic conditions

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Dedication

I dedicate my graduate work to my family. Without you, I would not have had the inspiration to pursue a career in scientific research. It has been your encouragement that has kept me going throughout the years. Thank you for the love and support you have given me.

Love always,

Meg

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Shigella flexneri ArcA and Fnr regulate iron acquisition and contribute to plaque formation under anaerobic conditions

Publication No._____

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Shigella flexneri is a Gram negative, intracellular pathogen responsible for bacillary dysentery in humans. To achieve infection of the human colonic epithelium, *S. flexneri* must adapt to varying environmental conditions, including fluctuations in pH, osmolarity, and nutrient and oxygen availabilities. The plaque assay is commonly used to measure the ability of *S. flexneri* to invade epithelial cells, grow intracellularly, and spread intercellularly to adjacent cells. However, as traditionally performed, this assay is of limited use in testing the virulence of *Shigella* in response to many of the conditions encountered in the host. I have modified the plaque assay to identify factors contributing to the virulence of *S. flexneri* under the anaerobic conditions present in the colon. This

assay demonstrated that the Feo transport system that acquires anaerobically abundant ferrous iron, as well as the transcription factors ArcA, Fnr, and Fur, impact *Shigella* plaque formation in anoxic environments. Transcriptional analyses indicated that anaerobic conditions activated expression of *feoABC* in *S. flexneri*. Anaerobiosis also repressed genes encoding two other iron transport systems that allow plaque formation by *S. flexneri* in aerobic environments, the ABC transporter Sit and the Iuc/Iut aerobactin siderophore synthesis and acquisition system that binds ferric iron, the dominant form of iron under aerobic conditions. The anaerobic regulators ArcA and Fnr induced expression of *feoABC*. Additionally, ArcA represses transcription of *iucABCDiutA* and *fur*. *fur* encodes a transcriptional regulator that is activated in the presence of iron and is responsible for repression of genes encoding iron acquisition systems and the sRNA RyhB that affects synthesis of iron-storage proteins including many TCA cycle enzymes. ArcA is a redox regulator known for redirecting metabolism upon oxygen depletion by down-regulating TCA cycle and aerobic respiratory enzymes and inducing fermentation and anaerobic respiratory complexes. However, ArcA regulation of *fur* and its downstream targets offers *S. flexneri* a means to coordinate energy and carbon metabolism with that of iron availability in response to environmental redox conditions.

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I. INTRODUCTION

1. Overview of *Shigella flexneri* pathogenesis

Shigellae are Gram negative, intracellular pathogens that cause bacillary dysentery and are responsible for approximately one million deaths each year (Kotloff *et al.*, 1999). They are divided into four species, *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei*, and each species contains numerous serotypes based on O-antigen variations (Jennison & Verma, 2004). *Shigella spp.* are similar to enteroinvasive *Escherichia coli* (EIEC) with respect to DNA sequences of housekeeping and virulence genes and are proposed to constitute a single pathovar of *E. coli* (Lan *et al.*, 2004). Distinct from other *E. coli*, EIEC and *Shigella* are intracellular pathogens. They are able to induce uptake into host epithelial cells due to the presence of a 220 kb virulence plasmid containing invasion genes (*ipa-mxi-spa*, *ipg*, *vir*, and *osp*). These genes encode a type III secretion system (TTSS) secretion apparatus, its secreted effectors, chaperones, and regulatory proteins (Venkatesan *et al.*, 2001).

Shigella spp. are highly infective pathogens, requiring as few as 10 to 100 organisms (Sansonetti, 2001), and are proficient in their abilities to adapt to a variety of environmental conditions. During host infection, shigellae encounter fluctuations in pH, osmolarity, and nutrient and oxygen availabilities. In the human, pathogens must

respond to a relatively constant temperature of 37°C. Some of these conditions are known to regulate the expression of virulence genes in enteric organisms including *Shigella*. For example, *Shigella* virulence is thermo-regulated. Exposure to 37°C signals bacterial entry into the host and induces the expression of the virulence gene regulators VirF and VirB, which are transcription factors responsible for the expression of the TTSS (Jost & Adler, 1993; Tobe *et al.*, 1995).

1.1. SHIGELLOSIS

Shigellosis, or bacillary dysentery, is an infection of the human colon by *Shigella* that is characterized by fever, intestinal cramps, and mucopurulent and bloody diarrhea. The public health burden of shigellosis in third-world countries is 100 times greater than that of industrialized nations, as it predominantly affects people in impoverished areas with poor sanitary conditions (Sansonetti, 2001). Since it affects approximately 150 million people annually and there is no effective vaccine, shigellosis and the etiological agents responsible for this disease are a priority focus of international public health research (Sansonetti, 2001).

During the course of infection (Fig. 1), *S. flexneri* crosses the epithelium by M cells surveying the lumenal contents of the intestine. Shigellae are deposited at a basolateral pocket enriched in macrophages (Wassef *et al.*, 1989). *S. flexneri* reaches the basolateral surface of the colonic epithelium after exiting the M cell, or if ingested by the macrophages, after inducing apoptosis. Phagocytized shigellae induce macrophage cell death through the actions of the invasion plasmid antigen B (IpaB) protein. IpaB,

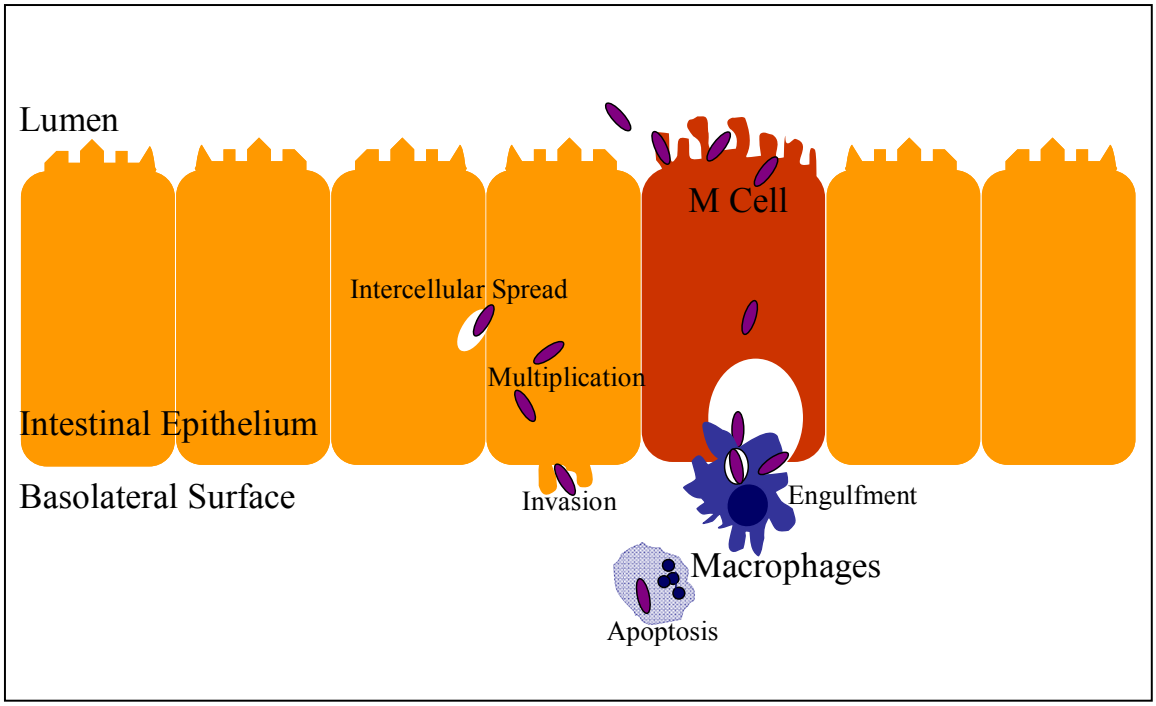


Figure 1. *S. flexneri* pathogenesis

After human ingestion, *S. flexneri* transits through the digestive tract and encounters M cells within the colonic epithelium. The M cells, which are involved in immune surveillance, sample the lumenal contents and deposit bacteria in a basolateral pocket where they are often engulfed by macrophages. After inducing apoptosis of macrophages, shigellae adhere to the basolateral surface of the epithelial cells and induce cytoskeletal changes. These changes promote uptake of the bacteria in vesicles. Shigellae escape the vesicle and multiply in the cytosol before spreading to adjacent epithelial cells.

encoded on the virulence plasmid, activates caspase 1 to cause apoptosis (Chen *et al.*, 1996), although necrosis is also stimulated by lipid A of lipopolysaccharide (LPS) (Suzuki *et al.*, 2005). These immune cells and the infected epithelial cells release cytokines such as IL-1β, IL-18, and IL-8 (Sansonetti, 2001). These cytokines promote the inflammatory response that results in the destruction of the epithelium. The tissue destruction allows increased bacterial access to the subepithelial space, as well as causing symptoms characteristic of shigellosis (Philpott *et al.*, 2000).

At the basolateral surface of the epithelial cells, *S. flexneri* entry is dependent on IpaB and IpaC. IpaC induces cytoskeletal reorganization that leads to membrane ruffling upon contact with the epithelial cell (Tran *et al.*, 2000). IpaC also acts with IpaB to form a pore that allows translocation of the other TTSS-secreted effector proteins into the epithelial cell (Blocker *et al.*, 1999). Many of these effectors, including IpaA and IpgD, also are involved in further reorganization of the epithelial cytoskeleton or post-invasion events (Sansonetti, 2001). *S. flexneri* is then engulfed in vesicles at the sites of membrane ruffling during a process called macropinocytosis. These engulfed bacteria induce lysis of the vesicles in an IpaB- and IpaC-dependent manner (High *et al.*, 1992), and are released into the cytoplasm. *S. flexneri* is able to divide within the cytoplasm and spreads directly to adjacent cells. This delays immune detection, increasing chances for bacterial survival within the host [reviewed in (Jennison & Verma, 2004; Ogawa & Sasakawa, 2006; Parsot, 2005)]. Intercellular spread is dependent on the action of polarly-localized IcsA, a virulence plasmid-encoded protein that promotes actin polymerization. The actin polymerization at the IcsA pole propels *S. flexneri* through the cell (Bernardini *et al.*, 1989). At the membrane, shigellae move into neighboring cells, which is dependent on Ipa secretion (Schuch *et al.*, 1999).

Ultimately, the immune system is responsible for clearing *S. flexneri* infection. The release of IL-18 by macrophages induces IFN-γ, which leads to macrophage and fibroblast activation and eradication of *S. flexneri* (Way *et al.*, 1998). IgA and IgG are produced upon primary exposure to *Shigella spp.* and provide serotype-specific immunity against subsequent infection (Mavris & Sansonetti, 2004). In addition to the role of the immune response in clearing shigellosis, antimicrobial therapy has proven effective in treatment of shigellosis, although multi-resistant strains of *Shigella spp.* are an emerging threat (Kotloff *et al.*, 1999).

1.2. MODELS OF SHIGELLA VIRULENCE

Because shigellosis is restricted to higher primates including humans, there is not a small animal model that truly assays *Shigella* virulence (Philpott *et al.*, 2000). Therefore, factors affecting *Shigella* pathogenesis are routinely investigated using cultured human epithelial cells. In confluent epithelial cell monolayers, plaques are formed by *S. flexneri* as they invade and spread intercellularly (Fig. 2). The size and number of plaques formed within these epithelial cell monolayers are used as indicators of virulence, since plaque formation measures the capacity of shigellae to invade, grow intracellularly, and spread directly to adjacent epithelial cells (Oaks *et al.*, 1985).

Polarized colonic epithelial monolayers are used in another cell culture model to measure *Shigella* virulence. In this model, the cells differentiate to mimic a columnar intestinal barrier (Mounier *et al.*, 1992). Because separate chambers allow exposure of

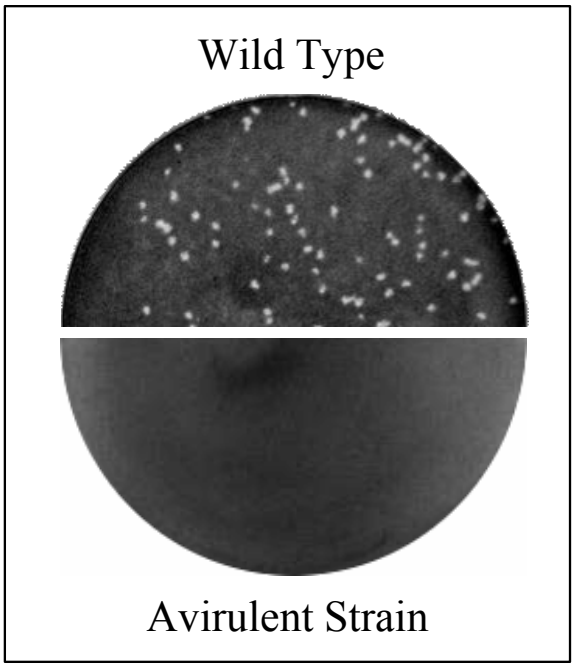


Figure 2. Plaque formation assays *Shigella* virulence

Infection of epithelial cell monolayers with *Shigella* leads to plaque formation. Sizes and numbers of plaques formed are used to determine the ability of bacteria to invade, multiply intracellularly, and spread into adjacent cells.

the bacteria to apical or basolateral surfaces, this model was integral in determining that exposure to the basolateral surface of the epithelium is required for *Shigella* infection (Mounier *et al.*, 1992). The polarized epithelial cell model has also been modified to incorporate polymorphonuclear leukocytes (PMNs), allowing investigation of *Shigella* infection of polarized epithelial cells in the presence of these immune cells. Observing these three cell types in concert led to elucidating the role of PMNs in disrupting intercellular gap junctions and increasing subepithelial infiltration by *Shigella* (Perdomo *et al.*, 1994a).

Although cell culture models of infection are unable to reproduce an immune response to infection or many of the complex variables that contribute to the microenvironments encountered during infection, they have provided a reliable means to screen for virulence determinants. A variety of animal models have also been used to examine aspects of *Shigella* infection. These animal models have provided insight into *Shigella*-host interactions and factors required for several stages of pathogenesis. The Serény test (Sereny, 1955) is performed by inoculating bacteria into the guinea pig or mouse kerato-conjunctival sac. The bacteria invade the conjunctival epithelia, producing conjunctivitis and keratitis. The Serény test is the standard model to determine the inflammatory response and host immunity for assessment of vaccine candidates.

Infection of ligated rabbit ileal loops also is used to investigate host responses to *Shigella* infection. During this procedure, the ileum is externalized and tied in sections. These sections are injected with bacteria and the intestine is returned to the abdominal cavity (Formal *et al.*, 1961). After 24 hours, the inflammatory response and tissue are analyzed. This model does not completely reflect host conditions, as there are

physiological differences between the ileum and colon. The process also elicits an acute immune response from the surgical procedure and makes the tissue more aerobic, evidenced by changes in microflora and histology (Keren *et al.*, 1980). However, the ileal loop experiments provide further evidence that cytokine secretion by macrophages is involved in the recruitment of PMNs, PMN transmigration increases bacterial access to the basolateral surface, and the acute inflammatory reaction leads to tissue destruction of the epithelium (Perdomo *et al.*, 1994a; Perdomo *et al.*, 1994b; Zychlinsky *et al.*, 1994).

Mice challenged intranasally with *S. flexneri* succumb to pneumonia, and some aspects of this infectious process mimics shigellosis (Voino-Yasenetsky & Voino-Yasenetskaya, 1962). *Shigella* invades the bronchial and tracheal epithelia, which exhibit similarities with the colonic epithelium. The pulmonary and intestinal epithelia contain ciliated columnar epithelial cells with lymphoid follicles, and upon infection, a massive immune response ensues that results in fluid accumulation (van de Verg *et al.*, 1995). The mouse lung model also has its limitations due to pulmonary and colonic differences in flora, oxygen availability, and pH. However, preliminary evidence indicates a high degree of correlation between infection of the human intestine and mouse lung regarding protection conferred against challenge and adverse affects, or reactogenicity, of strains (Mallett *et al.*, 1995). This assay, in conjunction with the Serény test, has been recommended for use in vaccine testing prior to challenge in human volunteers (Mallett *et al.*, 1995).

Currently, vaccine strains are often tested orally and by intraperitoneal administration in rhesus macaques, but this model is also not ideal for many reasons. The limitations of the macaque model include the requirement of a large inoculum (10^{10}

organisms compared with 10^1-10^2 in the human), inconsistent reactogenicity of strains between humans and monkeys, and logistical, financial, and ethical limitations (Philpott *et al.*, 2000).

2. *S. flexneri* Iron Acquisition

Many of the conditions present in the host environment affect the ability of *Shigella* to survive. Free iron is of limited availability within the human, as iron is tightly complexed with host proteins such as transferrin and lactoferrin (Bullen *et al.*, 1978), yet most organisms including *Shigella* require this trace metal for survival. Therefore, many pathogenic bacteria possess an arsenal of iron transport systems to compete with commensal organisms and the host for available iron and use complex regulatory mechanisms to maintain iron homeostasis (Andrews *et al.*, 2003). The presence of multiple iron transport systems that acquire different forms of iron allows for iron acquisition in diverse host environments. For example, oxidative bursts in macrophages and oxygen deprivation in the intestinal lumen test the ability of pathogens to acquire iron in different redox states.

Some host conditions also regulate virulence factors that mediate adherence, invasion, and intercellular spread. In *Shigella*, iron availability has been shown to directly influence the expression of virulence genes (Durand *et al.*, 1997; Durand *et al.*, 2000; Murphy & Payne, 2007; Sung *et al.*, 1990) and ability to cause infection in

epithelial cell and macrophage models of infection (Lucchini *et al.*, 2005; Nassif *et al.*, 1987; Runyen-Janecky *et al.*, 2006; Runyen-Janecky *et al.*, 2003). These findings further emphasize the significance of obtaining iron during *S. flexneri* pathogenesis.

2.1. IMPORTANCE OF MAINTAINING IRON HOMEOSTASIS

Iron exists in two oxidative states, ferric (Fe^{3+}) and ferrous (Fe^{2+}), under physiological conditions, and this property makes iron a suitable catalyst and electron carrier for a wide variety of enzymatic reactions. The importance of iron is evident in its requirement during diverse bacterial processes, including photosynthesis, respiration, H_2 production and consumption, the tricarboxylic acid (TCA) cycle, gene regulation, and DNA synthesis. Therefore, *E. coli* and related organisms maintain their iron content between 10^5-10^6 atoms per cell (Andrews *et al.*, 2003).

At pH 7, ferrous iron is relatively soluble (0.1 M), while ferric iron is extremely insoluble (10^{-18} M), forming iron oxides readily in the presence of oxygen (Weber *et al.*, 2006). However, as pH decreases, the formation of Fe^{2+} and the solubility of Fe^{3+} increase, and below pH 4, Fe^{2+} exists primarily in a soluble form regardless of oxygen availability (Weber *et al.*, 2006). Thus, during aerobic conditions above pH 4, bacteria must use available ferrous iron or solubilize ferric oxides to obtain sufficient iron to meet growth demands. Bacteria enhance iron solubilization by decreasing the external pH, reducing Fe^{3+} to Fe^{2+}, and secreting high affinity Fe^{3+} chelators called siderophores,

although the latter two processes are reported to be the primary mechanisms employed (Andrews *et al.*, 2003).

While iron is essential for growth, free iron can be toxic to cellular components after interaction with reactive oxygen species [Fig. 3; reviewed in (Imlay, 2002)]. Therefore, bacteria maintain a delicate balance between acquiring sufficient iron and guarding against iron toxicity. Superoxide (O_2^-), hydroxyl radicals (HO•), and hydrogen peroxide (H_2O_2) are reactive oxidants that damage proteins, lipids, and DNA. O_2^- and H_2O_2 are produced through the auto-oxidation of flavoproteins such as flavodoxin, reductases, and dehydrogenases involved in electron transfer reactions (Imlay, 1995; Messner & Imlay, 1999). Hydrogen peroxide reacts with Fe^{2+} to form hydroxide (OH^-) and hydroxyl (HO•) radicals (Fenton reaction) [Fenton, 1894; reviewed in (Halliwell & Gutteridge, 1984)]. Superoxide reacts with free Fe^{3+} and [4Fe-4S]-containing proteins, which liberates additional Fe^{2+} during oxidative stress and further propagates formation of hydroxyl radicals and hydrogen peroxide (Halliwell & Gutteridge, 1984). Reactive oxygen species damage enzymes by oxidizing cysteinyl residues (Aslund *et al.*, 1999; Ellis & Poole, 1997), destroying iron-sulfur clusters (Flint *et al.*, 1993; Kuo *et al.*, 1987), and creating protein adducts (Dukan & Nystrom, 1999; Dukan *et al.*, 2000; Levine *et al.*, 1996). Reactive oxygen species also damage membranes (Gutteridge & Halliwell, 1990). However, most detrimental are the heritable mutations in the genome caused by HO•-mediated DNA lesions (Hutchinson, 1985). In response to this damage, bacteria produce enzymes for DNA repair, iron-sulfur cluster assembly, and degradation of reactive oxygen species. Many of these enzymes are regulated in response oxidative damage, as well as the availabilities of iron and oxygen [reviewed in (Green & Paget, 2004)]. Additionally, expression of bacterial iron uptake systems is coordinated with the

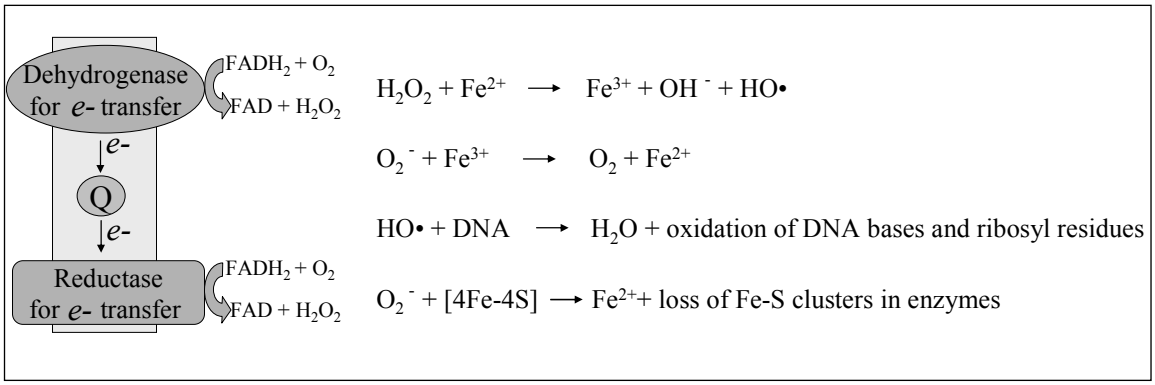


Figure 3. Oxygen-induced iron toxicity

Flavoproteins of respiratory dehydrogenases and reductases react with molecular oxygen (O_2) to produce hydrogen peroxide (H_2O_2) and superoxide (O_2^-). O_2^- reacts with ferric iron to form ferrous iron, while H_2O_2 reacts with ferrous iron to produce the highly reactive oxygen species hydroxide (OH^-) and the hydroxyl radical (HO•). HO• and O_2^- lead to DNA damage and inactivation of enzymes.

intracellular iron content and redox status to decrease the accumulation of iron when its acquisition would be deleterious.

2.2. IRON TRANSPORTERS OF *S. FLEXNERI*

Shigella species have multiple mechanisms to acquire iron (Fig. 4), although the presence of these iron transport systems varies by species, and even within a given serotype (Payne & Mey, 2004). All *Shigella* species use the Feo ferrous iron transport system, which is ubiquitous among enterics, and the Sit ABC transporter that is predicted to mediate uptake of manganese and iron (Payne & Mey, 2004). *S. flexneri* also synthesizes and transports the siderophore aerobactin (Payne, 1980), and has a transport system for the fungal siderophore ferrichrome (Dolence *et al.*, 1991). In addition, certain *S. flexneri* strains synthesize the Fec transporter for uptake of ferric-citrate, but this system is not functional in the wild type *S. flexneri* 2a strain SA100 studied in our laboratory.

2.2.1. Ferric Iron Acquisition

Aerobactin is a hydroxamate siderophore that contains a citrate molecule linked to two lysine residues, each with an acetyl group donated from acetyl Co-A (Walsh & Marshall, 2004). The genes encoding the enzymes responsible for aerobactin synthesis

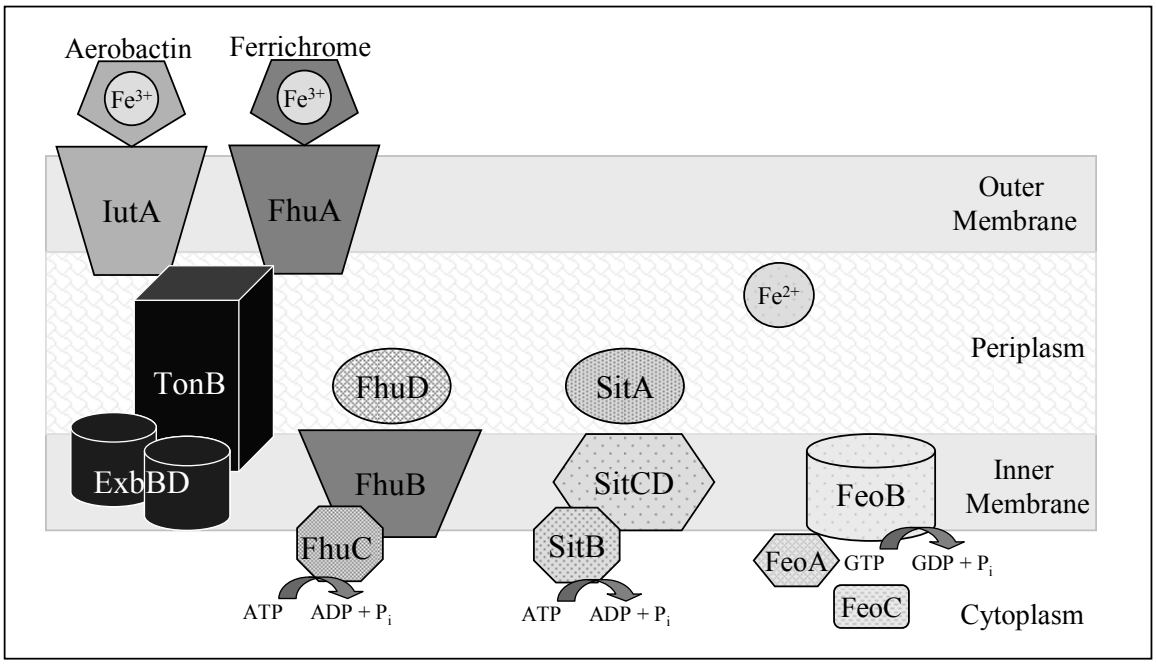


Figure 4. Iron acquisition systems of *S. flexneri*

S. flexneri uses endogenous (aerobactin) and exogenous (ferrichrome) siderophores to obtain Fe^{3+}. These ferri-siderophores bind the IutA and FhuA outer membrane receptors, which are energized for transport by contact with the TonB-ExbB-ExbD system. Once in the periplasm, the ferri-siderophores bind FhuD and are shuttled to the FhuB siderophore permease. FhuC hydrolyzes ATP to enable the siderophores to cross the inner membrane through FhuB (Andrews *et al.*, 2003). *S. flexneri* also transports soluble Fe^{2+}. SitA is predicted to shuttle Fe^{2+} in the periplasm to the SitCD integral membrane permease that is energized by SitB hydrolysis of ATP (Runyen-Janecky *et al.*, 2003). The FeoABC is known to be involved in Fe^{2+} transport. FeoB is believed to be a GTPase that translocates Fe^{2+} to the cytoplasm. Based on protein homologies, FeoA and FeoC are cytoplasmic accessory and regulatory proteins, respectively (Cartron *et al.*, 2006).

(*iucABCD*) and the TonB-dependent outer membrane receptor for its transport (*iutA*) are clustered in an 8.3 kb operon with a single promoter upstream of *iucA* (Fig. 5). This locus is contained within pathogenicity islands in *S. flexneri* and *S. boydii* (Lawlor *et al.*, 1987; Purdy & Payne, 2001; Vokes *et al.*, 1999). While a single polycistronic mRNA is transcribed, complex stem-loop structures and differences in codon usage account for varied levels of these proteins (Martinez *et al.*, 1994).

Previous findings by our laboratory demonstrate that the aerobactin genes are repressed during intracellular growth of *Shigella* (Headley *et al.*, 1997). However, aerobactin transport is important for the growth of *S. flexneri* within extracellular tissues in a ligated ileal loop model of infection (Nassif *et al.*, 1987), suggesting its importance during certain stages of enteric pathogenesis.

2.2.1.1. Energizing Iron Transport across the Outer Membrane

Since the outer membrane of Gram negative bacteria lacks access to ATP or an ion gradient to energize transport, ferric siderophores cross the outer membrane with the help of the TonB-ExbB-ExbD complex (Postle, 1990). The TonB complex facilitates transport by transferring energy from proton motive force (PMF) in the cytoplasmic membrane to the high-affinity receptors at the outer membrane (Postle, 1990). TonB is anchored in the cytoplasmic membrane with accessory proteins ExbB and ExbD, which are thought to create a channel for protons (Zhai *et al.*, 2003). TonB extends through the periplasmic space to the outer membrane, where it makes direct contact with the TonB-

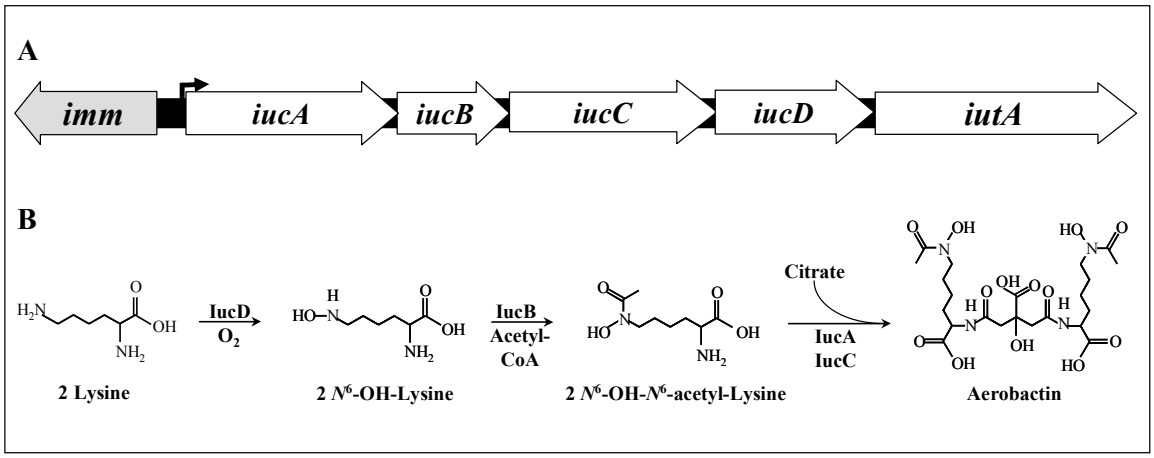


Figure 5. Aerobactin biosynthesis and genetic organization

A. The aerobactin biosynthesis and transport locus includes the *iucABCD* genes for synthesis of aerobactin from lysine and citrate and *iutA* encoding the aerobactin outer membrane receptor.

B. The pathway of aerobactin biosynthesis from lysine and citrate is depicted. The first step requires O_2 for IucD mono-oxygenase activity [modified with permission from (Davies, 2006)].

dependent receptors (Heller *et al.*, 1988). This contact mediates a conformational change in the receptors that appears to unplug them, allowing active transport of the ligands bound to the receptors (Locher *et al.*, 1998).

2.2.1.2. Periplasmic and Inner Membrane Transport of Ferri-siderophores

Once the hydroxamate siderophores such as aerobactin cross the outer membrane to the periplasm through their respective TonB-dependent receptors, they require the ABC transporter FhuBCD to gain access to the cytoplasm. The iron-bound siderophore interacts with the periplasmic binding component, FhuD (Fig. 4) (Carter *et al.*, 2006). FhuD transfers the iron-siderophore complex to the FhuB inner membrane permease that makes a channel for transport across the cytoplasmic membrane (Koster & Braun, 1989). FhuC is an ATPase associated with the permease that supplies energy for the ferri-hydroxamate transport (Burkhardt & Braun, 1987).

The genes encoding the FhuBCD transporter are located within an operon that also contains the *fhuA* gene for the ferrichrome receptor (Fecker & Braun, 1983). The enteric pathogens *E. coli*, *Salmonella*, and *Shigella* use the TonB-dependent FhuA receptor for uptake of Fe^{3+} complexed with the hydroxamate siderophore ferrichrome (Payne & Mey, 2004). Ferrichrome synthesis has only been reported by members of the corn smut fungus *Ustilago* (Ecker *et al.*, 1982; Wang *et al.*, 1989), but ferrichrome-like molecules are produced by fungi including *Aspergilli*, *Pennicillia*, *Neurospora*, and *Ustilago* (Neilands, 1981).

Since an environmental lifecycle for *S. flexneri* absent the host has not been reported, it is not clear why the *fhuA* gene for fungal siderophore acquisition is maintained. However, a single promoter controls *fhuACDB* expression. Interestingly, this promoter was more active intracellularly compared with its level during growth in Luria-Bertani (LB) broth (Runyen-Janecky & Payne, 2002). The intracellular increase of *fhuA* promoter activity may reflect the presence of an unidentified ligand that may gain entry via the FhuA transporter during growth within the host. In the absence of an additional substrate that could enter through this receptor, the loss of *fhuA* could prevent transcription of the downstream genes encoding the ABC transporter that enables siderophore iron acquisition. Because siderophore-dependent iron uptake through FhuBCD is important for pathogens, the ability to withstand iron restriction within the host environment may be compromised in the event of a loss of *fhuA*.

2.2.2. Other Iron Acquisition Systems

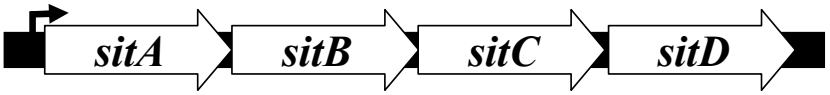
Ferrous iron transport systems are used by bacteria predominantly during growth in environments that are anaerobic or otherwise reducing (Kammler *et al.*, 1993). It is postulated that this form of iron uptake predates that of ferric iron transport since primitive environmental conditions on the earth were oxygen-poor until the advent of photosynthesis. *S. flexneri* has a homologue of the Sit metal-type ABC transport system that is also capable of ferrous iron transport in other organisms (Bearden & Perry, 1999; Kehres *et al.*, 2002; Sabri *et al.*, 2006), although it has not been experimentally

determined which form of iron is transported by Sit in *Shigella*. *S. flexneri* also contains a homologue of the Feo system for ferrous iron transport.

2.2.2.1. The Sit ABC Transport System

Based on *in vivo* and *in vitro* studies and its homology with other systems (Runyen-Janecky *et al.*, 2003), the *Shigella* Sit system appears to be an ABC transporter that is capable of functioning in iron acquisition (Fig. 6). Similar to other ABC transporters, this system is predicted to contain a periplasmic binding protein (SitA) that shuttles its substrate from the periplasmic space to the inner membrane permease (SitCD). The SitCD permease harnesses energy from hydrolysis of ATP by an associated ATPase (SitB). SitB energizes transfer of the substrate from the periplasm to the cytoplasm (Fig. 4). The *Shigella* Sit system exhibits significant homology to the Sit systems of APEC, *Salmonella enterica* serovar Typhimurium, *Sinorhizobium meliloti*, and *Agrobacterium tumefaciens* and to the Yfe systems of *Haemophilus influenzae*, *Yersinia pestis*, and *Pasteurella multocida*, with the greatest similarity to the APEC Sit system (Sabri *et al.*, 2006). The APEC Sit system functions in uptake of Mn^{2+}, Fe^{2+}, and Fe^{3+}, although it has a stronger affinity for Fe^{2+} than for Mn^{2+} or Fe^{3+} (Sabri *et al.*, 2006). Evidence also suggests the *S. flexneri* Sit system is capable of transporting both manganese and iron. Growth defects of the *sitA* mutant in the presence of the iron-chelating agent ethylene diamino-*o*-dihydroxyphenyl acetic acid (EDDA) can be restored by addition of either metal to growth medium (Runyen-Janecky *et al.*, 2006; Runyen-Janecky *et al.*, 2003). In addition, the *S. flexneri feo iuc sit* mutant is incapable of plaque

A



B

Homologues of *S. flexneri* SitABCD	% Identity to SitA	SitB	SitC	SitD
Avian pathogenic *E. coli* (APEC) Sit	98	98	99	96
S. enterica serovar Typhimurium Sit	77	77	75	64
S. meliloti Sit	70	70	68	55
A. tumefaciens Sit	65	67	65	49
H. influenzae Yfe	67	60	55	46
Y. pestis Yfe	64	61	59	47
P. multocida Yfe	62	58	53	46

Figure 6. Sit operon and homologous proteins

A. The *S. flexneri sitABCD* genes are co-transcribed from a single promoter.

B. Homologies of the *S. flexneri* Sit proteins with the Sit and Yfe Mn^{2+} and Fe^{2+} transport proteins of other organisms.

formation *in vivo*, which indicates that this mutant is unable to transport iron in the intracellular environment (Runyen-Janecky *et al.*, 2003).

The Sit system is prevalent among intracellular pathogens, which suggests a role for Sit during replication of pathogens within host cells. Further, the *S. flexneri sit* genes have been shown to be de-repressed upon entry into epithelial cells and macrophages (Lucchini *et al.*, 2005; Runyen-Janecky & Payne, 2002). Transcription of *sit* is repressed in the presence of high levels of iron and manganese, and the intracellular environment is thought to be restricted in both of these metals. Thus, it is likely that the low levels of manganese and iron within these cells triggers the de-repression of the *sit* locus. The loss of Sit also correlates with decreased survival of *S. flexneri* and other pathogens upon exposure to oxidative stress *in vitro* and within macrophages (Davies & Walker, 2007; Lucchini *et al.*, 2005; Runyen-Janecky *et al.*, 2006; Sabri *et al.*, 2006). Bacteria respond to oxidative stress by synthesizing superoxide dismutase, which catalyzes the reaction of superoxide and hydrogen ions to form water and hydrogen peroxide. Superoxide dismutases of *Shigella* require manganese or iron cofactors, and evidence suggests these cofactors are provided, in part, by the Sit system. Thus, the prevalence of the *sit* genes among invasive pathogens may reflect the role of Sit in Mn^{2+} or Fe^{2+} acquisition to withstand oxidative bursts in macrophages, as well as its ability to acquire Fe^{2+} for various metabolic processes.

2.2.2.2. The Feo Ferrous Iron Transport System

The Feo system (Fig. 7) is a widely distributed Fe^{2+} transporter with homologues in archaea and both Gram positive and Gram negative bacteria (Andrews *et al.*, 2003). This system was the first bacterial ferrous iron acquisition system identified (Kammler *et al.*, 1993), yet little is known about its mechanism of action. Like *E. coli* FeoB, *S. flexneri* FeoB is predicted to be a 773 amino acid, cytoplasmic membrane-associated protein with N-terminal GTPase activity and 7-12 putative transmembrane domains at the C-terminus. In most species, the *feoB* gene is found adjacent to the *feoA* gene. *feoA* encodes a 75 amino acid hydrophilic protein in *E. coli* and *S. flexneri*. FeoA is presumed to be a cytoplasmic protein that binds FeoB to mediate its activity via an SH3-like domain. SH-3 domains in eukaryotes often participate in interactions with G-proteins for processes such as localization and modulating GTPase function. A third open reading frame (ORF), *feoC*, is found in *feo* operons of *E. coli*, *Shigella* and several other γ-proteobacteria. In *Shigella* and *E. coli*, *feoC* is predicted to encode a 78 amino acid, cysteine-rich protein predicted to contain an Fe-S cluster. Since FeoC contains an N-terminal domain with similarity to winged helix motifs, it is believed to be a DNA binding protein whose regulatory function, like that of other Fe-S transcriptional regulators, is sensitive to redox conditions (Cartron *et al.*, 2006).

The Feo iron transport system has recently been shown to be important for intracellular survival and virulence of a number of pathogenic bacteria, including *Legionella pneumophila* (Cianciotto, 2007; Robey & Cianciotto, 2002), *Campylobacter jejuni* (Naikare *et al.*, 2006), *Helicobacter pylori* (Velayudhan *et al.*, 2000), and *S. enterica* serovar Typhimurium (Boyer *et al.*, 2002; Tsolis *et al.*, 1996). Studies by our laboratory have also demonstrated the importance of the Feo Fe^{2+} acquisition system in

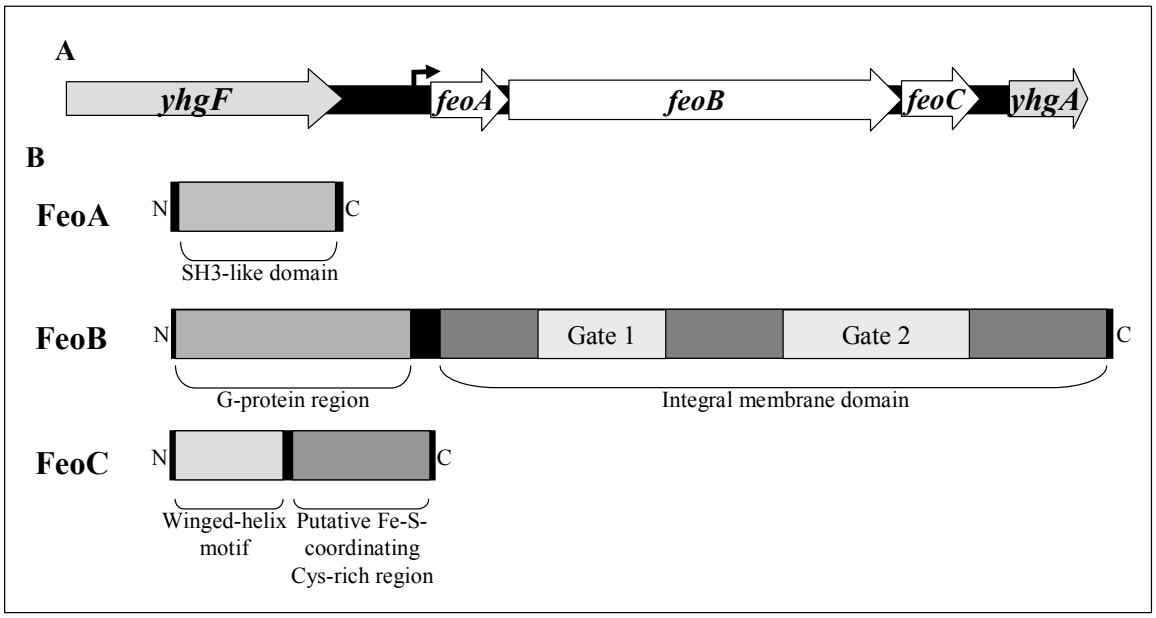


Figure 7. Genetic organization and protein domains of the *S. flexneri* Feo system

A. The *feoABC* genes are found within a 2.8 kb operon and encode proteins of 75, 773, and 78 amino acids, respectively.

B. FeoA contains a region similar to SH3 domains that are found in proteins that assist in the localization or function of GTPases. FeoB has an N-terminal region with homology to G-proteins and a C-terminal domain that localizes it to the cytoplasmic membrane. This integral membrane domain also has two Gate motifs that are characteristic of nucleoside transporters, and a similar arrangement has been found in the Ftr1p iron permease of yeast (Cartron, *et al*., 2006). FeoC contains an N-terminal winged-helix, typically used for DNA binding, and a C-terminal region with four cysteine residues, often associated with iron-sulfur clusters.

the virulence of *Shigella*, as *S. flexneri feo* mutants lacking either Sit or the aerobactin system form small plaques in epithelial cell monolayers (Runyen-Janecky *et al.*, 2003).

2.3. Regulatory Mechanisms for Maintaining Iron Homeostasis

Obtaining sufficient iron to meet metabolic demands is important for the growth and pathogenicity of *S. flexneri*, while ensuring that excess free iron is not present intracellularly reduces the risk of cytotoxicity of iron oxidative stress. This delicate iron equilibrium is maintained by several levels of regulation on the expression of genes involved in iron acquisition, iron storage, and iron-sulfur cluster assembly. Even the regulatory factors that ensure proper iron homeostasis are subject to complex regulation in response to diverse signals.

2.3.1. Regulation of Iron Acquisition by Iron Availability and Fur

The intracellular iron concentration directly influences the expression of iron acquisition genes by a negative feedback mechanism involving an iron-binding transcriptional regulator called Fur (Bagg & Neilands, 1987). Fur is a dimeric DNA binding protein whose affinity for specific DNA sequences is increased 1000-fold upon association with Fe^{2+} (De Lorenzo *et al.*, 2004). Thus, during iron limitation Fur has little effect on mediating transcriptional regulation. However, when the cell becomes iron loaded, Fur binds iron and Fe-Fur binds to regulatory elements, Fur boxes, found

upstream of genes repressed by Fur (Fig. 8). Fe-Fur association with DNA blocks RNA polymerase from binding, and initiation of transcription is prevented (Escolar *et al.*, 1997; Escolar *et al.*, 1998). In *S. flexneri* the genes directly repressed by Fur include, among others, those encoding the Iut and Iuc proteins for the biosynthesis and uptake of aerobactin, the Fhu, Sit, and Feo iron transport systems, and a small RNA (sRNA) known as RyhB (Oglesby *et al.*, 2005). Therefore, Fur serves as an intracellular iron sensor to shut down iron acquisition when metabolic demands are exceeded and additional uptake would prove deleterious to the cell.

2.3.2. RyhB Regulation of Fe-S Cluster Assembly and Iron Storage

Excessive iron acquisition and the instability of iron-sulfur clusters during oxidative stress can cause an overabundance of free iron and result in oxidative damage (McCormick *et al.*, 1998; Touati *et al.*, 1995). During iron abundance, Fe-Fur represses transcription of the sRNA RyhB (Masse & Gottesman, 2002). RyhB is a non-coding RNA that promotes degradation of target transcripts (Fig. 8) (Masse & Gottesman, 2002; Masse *et al.*, 2003; Masse *et al.*, 2005). RyhB destabilizes transcripts or blocks translation of ferritin-like proteins whose primary function is to sequester iron. It also regulates the synthesis of proteins that contain iron as a cofactor. The latter group includes proteins that participate in diverse processes such as aerobic and anaerobic respiration, the TCA cycle, and fermentation. Fe-Fur repression of RyhB therefore increases iron storage, and further, ties energy and carbon metabolism to iron availability. It is through this regulation that Fur indirectly increases the synthesis of iron storage

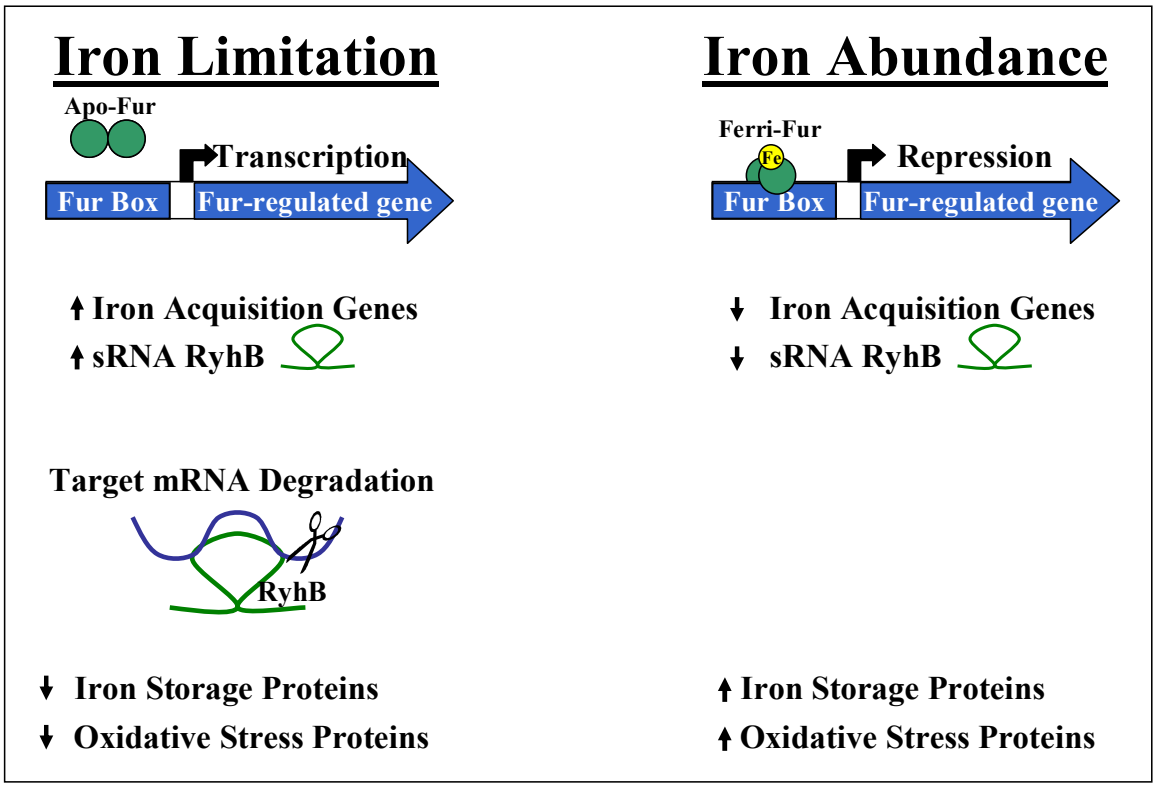


Figure 8. Iron availability impacts gene regulation by Fur and RyhB

During iron limitation, iron acquisition genes are transcribed, allowing iron uptake. RyhB, a repressor of iron storage and oxidative stress proteins, is also made in low iron. When iron is abundant, Fe^{2+} binds Fur dimers and increases the affinity of ferri-Fur for Fur boxes. Ferri-Fur represses genes encoding iron acquisition and the synthesis of RyhB. Repression of RyhB permits the synthesis of iron storage and oxidative stress proteins, which counteracts iron toxicity that results from excess iron accumulation.

proteins and enzymes that promote the stability and re-formation of Fe-S clusters during oxidative stress to reduce the amount of free iron present in the cell.

2.3.3. Regulation of Fur

Iron availability not only influences the activity of Fur, but also regulates the transcription of *fur* due to auto-repression by Fe-Fur (De Lorenzo *et al.*, 1988; Delany *et al.*, 2002; Hernandez *et al.*, 2006; Sala *et al.*, 2003). Additionally, the expression of *fur* is decreased in strains with mutations in *crp,* encoding the cyclic AMP (cAMP) receptor protein (Crp), or *cya*, encoding the adenylate cyclase enzyme that generates cAMP (De Lorenzo *et al.*, 1988). Because cAMP levels increase during growth with carbon sources other than glucose, the source of cellular carbon impacts iron uptake (De Lorenzo *et al.*, 1988). The redox regulators activated by oxidative stress, OxyR and SoxS, also fine-tune Fur levels by activating *fur* transcription (Aslund *et al.*, 1999). An increase in Fur synthesis provides the cell a means to sequester unbound iron while turning off iron acquisition, thereby preventing the production of cell-damaging radicals that are generated by Fenton chemistry during growth.

In addition to transcriptional regulation of *fur*, two mechanisms of post-transcriptional repression of *fur* in response to low iron levels have been identified (Vecerek *et al.*, 2007). The first mechanism is RyhB-dependent, while the second is tRNA-dependent. In *E. coli*, an ORF of 28 codons immediately upstream of *fur* (*uof*) is transcribed with *fur*. A binding site for RyhB is found within the *uof* mRNA, and RyhB

directs degradation of the transcript during iron limitation. The *uof* transcript also contains the serine codon UCA at the sixth position immediately followed by an AGA codon. UCA is decoded by a rare, modified $tRNA^{Ser}_{IV}$ that is synthesized in an iron-dependent pathway involving the MiaA and MiaB proteins (Connolly & Winkler, 1989). Recently it was demonstrated that the MiaB protein is an iron-sulfur cluster protein. Therefore, iron is required for the synthesis of the 2-methylthio-N^6-(Δ^2-isopentyl)-adenosine-37 (ms^2i^6A-37) modification of the $tRNA^{Ser}_{IV}$ (Pierrel *et al.*, 2002). The AGA codon of *uof* corresponds to the rare $tRNA^{Arg}_4$, and its presence near the initiation codon leads to ribosome stalling and mRNA release (Cruz-Vera *et al.*, 2004). Therefore, during iron limitation, the presence of these two adjacent codons near the translational start of the *uof-fur* mRNA causes ribosomal stalling and inhibits translation of *fur*.

2.4. IRON ACQUISITION *IN VIVO*

The expression of iron acquisition systems is important for plaque formation in epithelial cell monolayers by *Shigella* (Payne *et al.*, 2006; Runyen-Janecky *et al.*, 2003). Iron uptake by *S. flexneri* within epithelial cells or during growth in laboratory conditions is achieved through the Sit, Feo, and aerobactin transport systems, as a *feo iuc sit* mutant is unable to grow in either environment in the absence of exogenously supplied siderophore (Runyen-Janecky *et al.*, 2003). Feo, Sit, and siderophore iron acquisition systems also impact the virulence of other human pathogens, including *S. enterica* serovar Typhimurium, *L. pneumophila*, *H. pylori*, *C. jejuni*, and *E. coli,* and numerous Gram positive bacteria (Boyer *et al.*, 2002; Brown & Holden, 2002; Cianciotto, 2007;

Naikare *et al.*, 2006; Robey & Cianciotto, 2002; Tsolis *et al.*, 1996; Velayudhan *et al.*, 2000), suggesting that the ability to acquire iron with multiple transport systems during infection is a trademark of human pathogens and essential to withstand the host environment.

Iron acquisition is significant for the survival of pathogens within the host; however, the virulence genes of *Shigella spp.* are also regulated by iron availability. The classic example of virulence gene regulation by iron availability is the Fur-mediated repression of *S. dysenteriae* Shiga toxin production (Calderwood & Mekalanos, 1987). However, virulence genes are also induced by the presence of iron in *Shigella spp.* In *S. dysenteriae*, RyhB represses the synthesis of the VirB transcription factor that lies just downstream of the VirF master regulator in the regulatory cascade that activates the *ipa-mxi-spa* genes required for virulence (Murphy & Payne, 2007). Additionally, the same $tRNA^{Ser}_{IV}$ that affects translation of Fur also is required for translation of VirF in *S. flexneri* (Durand *et al.*, 1997; Durand *et al.*, 2000). Regulation of VirB and VirF by these two iron responsive factors ensures that the TTSS is only expressed when *Shigella* has acquired sufficient iron for growth.

3. Anaerobiosis as a Regulatory Signal for Iron Acquisition and Bacterial Pathogenesis

The lumen of the colon has limited oxygen availability as evidenced by the fact that 99.9% of the microflora that colonize this area are obligate anaerobes (Hao & Lee, 2004). Oxygen limitation has been shown to play a role in the persistence and virulence of many pathogenic bacteria, including enteric pathogens such as *E. coli* (Diard *et al.*, 2006; James & Keevil, 1999), *Salmonella spp.* (Contreras *et al.*, 1997; Kapoor *et al.*, 2002; Khullar *et al.*, 2003; Singh *et al.*, 2000), *Vibrio cholerae* (Bina *et al.*, 2003; Sengupta *et al.*, 2003; Xu *et al.*, 2003), and *Yersinia enterocolitica* (Schmiel *et al.*, 2000). Given that *Shigella spp.* invade and reside within the colon during infection, anaerobic metabolism may also be important during *Shigella* pathogenesis. Anaerobiosis may serve as a signal to *Shigella* that the site of colonization has been reached and induce expression of virulence genes that are required for epithelial invasion or intercellular dissemination. Additionally, the ability to regulate iron transport in response to changes in oxygen within the host may be essential for *Shigella* to persist in this environment.

3.1. OXYGEN AVAILABILITY AND METABOLISM

Anaerobic conditions influence bacterial metabolism by transcriptional repression and enzymatic inactivation of factors involved in aerobic energy production via aerobic respiration and a functional TCA cycle. During anaerobiosis, genes necessary for anaerobic survival are induced. These include genes required for fermentation, branched Krebs biosynthetic pathways, and anaerobic respiration. More ATP is evolved by proton translocation through the F_1F_0 ATPase during anaerobic respiration than by substrate-level phosphorylation during fermentation. Therefore, respiration is favored in the

presence of anaerobically available electron donors, including H_2, lactate, formate, α-glycerol-phosphate, and NADH, and acceptors such as nitrate, nitrite, fumarate, dimethyl sulfoxide (DMSO), and trimethylamine oxide (TMAO) (Moat *et al.*, 2002; Unden & Bongaerts, 1997).

3.2. ANAEROBIC TRANSCRIPTION FACTORS

Anaerobic adaptation by *E. coli* and other bacterial species is mediated primarily by the two-component ArcAB (anoxic redox control) system and the transcription factor Fnr (fumarate and nitrate reduction). In *E. coli*, there are additional factors that regulate genes in response to anaerobic conditions, including Aer, FlhDC, and CitAB (Pruss *et al.*, 2003; Sawers, 1999). The *arcA*, *arcB*, and *fnr* genes are predicted to encode fully functional proteins in *S. flexneri*; however, *aer*, *citB*, and *flhD* are annotated as pseudogenes in the *S. flexneri* genome sequences (Jin *et al.*, 2002; Wei *et al.*, 2003). Therefore, the regulators encoded by these genes are not predicted to be operative in this organism (Jin *et al.*, 2002; Wei *et al.*, 2003), suggesting that ArcAB and Fnr are the primary anaerobic regulators in *S. flexneri*.

3.2.1. ArcA

The *E. coli arcA* gene was initially found mutated in strains that displayed increased sensitivity to redox dyes (*dye*) and male-specific phage (*msp*) resistance

(Buxton *et al.*, 1978; Roeder & Somerville, 1979). *arcA* was also referred to over the years as *fex*, for F expressionless (Lerner & Zinder, 1979), *sfrA*, for sex factor regulation (Beutin & Achtman, 1979), and *cpxC*, for conjugative plasmid expression (McEwen & Silverman, 1980). Ten years after its discovery, the acronym *arcA* was finally given to this gene for aerobic respiration control since the anaerobic activities of various metabolic enzymes were increased by mutation of *arcA*, including those of the TCA enzymes, numerous dehydrogenases, isocitrate lyase, and ubiquinol oxidase (Iuchi & Lin, 1988). It was noted that the *arcA* mutant had a phenotype similar to that of the previously characterized, unlinked *arcB* mutant and that ArcA exhibited similarity to the OmpR regulator of outer membrane proteins. It was further suggested that perhaps ArcA was a soluble transcriptional regulator that worked in global metabolic regulation in conjunction with the membrane bound ArcB, which was later verified (Iuchi & Lin, 1988).

The ArcAB two-component signal transduction system is the primary factor regulating the bacterial transition to anaerobiosis. It is composed of ArcA, the cytoplasmic response regulator, and ArcB, the membrane-associated sensor that detects anaerobiosis and activates ArcA (Fig. 9). Anaerobiosis is detected by the accumulation of reduced quinones in the membrane, and these quinols lead to autophosphorylation of the ArcB sensor, which then becomes a kinase to activate ArcA and increase its specificity for target DNA sequences (Georgellis *et al.*, 2001; Malpica *et al.*, 2004; Malpica *et al.*, 2006).

ArcB is a histidine kinase with two transmembrane domains for localization near the quinones, a large cytoplasmic phosphotransfer region containing three catalytic

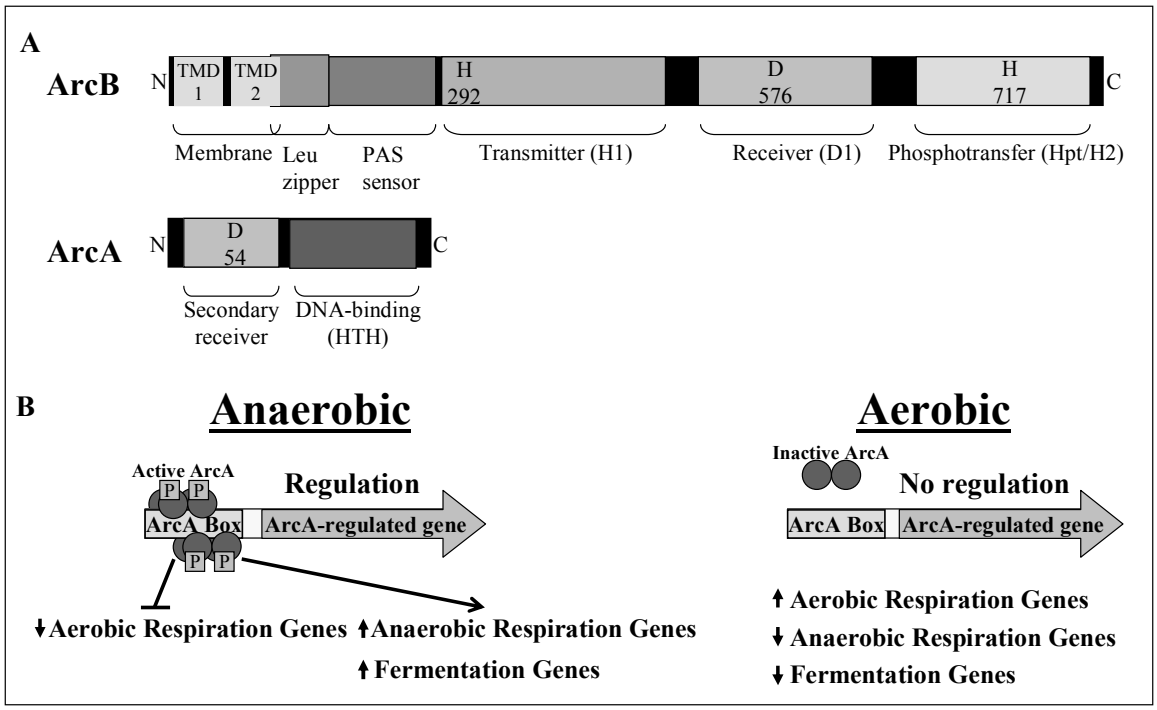


Figure 9. Protein domains of and regulation by ArcAB signal transduction system

A. ArcB is a histidine kinase with four N-terminal regions allowing it to monitor redox status: two transmembrane domains (TMD) for membrane insertion, a leucine zipper for self-association, and a PAS domain with cysteine residues for sensing reduced quinones in the membrane. Its C terminus is comprised of H1, D1, and Hpt catalytic regions involved in His-Asp-His phosphotransfer reactions. ArcA has an N-terminal receiver domain that is phosphorylated by ArcB in the presence of reducing conditions, which leads to conformational changes in the C-terminal helix-turn-helix (HTH) region, increasing its affinity for DNA regions called ArcA boxes.

B. During anaerobic, reducing conditions, ArcA binds promoters to repress or induce target genes, including those involved in respiration and fermentation.

domains (Malpica *et al.*, 2004). It contains only a small periplasmic loop region between the transmembrane domains, making it unique among two-component sensors that typically have large periplasmic regions for signal sensing (Malpica *et al.*, 2004). These characteristics classify ArcB within a tripartite hybrid sensor kinase subfamily of histidine kinases that participate in multiple phosphoryl reactions involving a His-Asp phosphorelay (Kwon *et al.*, 2000).

ArcA is a member of the OmpR/PhoB subfamily of response regulator transcription factors (Toro-Roman *et al.*, 2005). This is the largest subfamily of response regulators, which bind as tandem dimers (tetramers) to direct repeat recognition sequences and are distinguished by a C-terminal winged helix-turn-helix DNA binding motif. ArcA was the first of this family of response regulators for which a crystal structure was available. The crystal structure indicates that dimerization by phosphorylation of the N-terminal regulatory region, in conjunction with additional dimerization mediated by the DNA binding region, allows ArcA to multimerize into tetrameric and even octameric structures (Jeon *et al.*, 2001; Toro-Roman *et al.*, 2005).

Although consensus sequences for the site recognized by ArcA have been reported in the literature, the optimal consensus for predicting ArcA binding is not known (Drapal & Sawers, 1995; Favorov *et al.*, 2005; Liu & De Wulf, 2004; Lynch & Lin, 1996; McGuire *et al.*, 1999; Minagawa *et al.*, 1990). Recent transcriptional profiling of an *E. coli arcA* mutant and subsequent comparisons to a new ArcA recognition weight matrix allowed identification of novel ArcA targets (Liu & De Wulf, 2004). In addition to its roles as a repressor of aerobic metabolic genes and activator of fermentative metabolic genes, ArcA now appears to regulate a wide variety of processes. The

biosynthesis and metabolism of amino acids, phospholipids, and nucleic acids, transport of cofactors and sugars, synthesis of cell structures such as curli fibers and flagella, the oxidative stress response, and transposon and phage related factors are regulated by ArcA. ArcA has also been shown to regulate bacterial virulence, as *V. cholerae* and *H. influenzae arcA* mutants were 100-fold and 15-fold less virulent in animal models, respectively (De Souza-Hart *et al.*, 2003; Sengupta *et al.*, 2003).

3.2.2. Fnr

Initially called *frdB* for its role in fumarate reductase activity (Bachmann *et al.*, 1976), the *fnr* mutant of *E. coli* was reported to have combined defects in fumarate and nitrate reduction (Lambden & Guest, 1976). There was no growth of this mutant anaerobically on minimal medium containing glycerol, lactate, or formate with fumarate or nitrate, although growth was observed on glucose minimal medium (Lambden & Guest, 1976). The activities of fumarate and nitrate reductases in the *fnr* mutant were reduced to ten percent or less of wild type levels. It was inferred that this class of mutants must have a defect in some common component essential for fumarate and nitrate respiration systems such as a cytochrome, or a mutation in a regulatory or structural gene. Fnr is now known to be a global anaerobic regulator that induces the fumarate and nitrate reductases, and numerous studies have been undertaken to determine how this transcriptional regulator functions and the targets of its regulation.

Fnr (Fig. 10) is a founding member of the Crp-Fnr superfamily of transcriptional regulators. The Fnr group of proteins is further characterized by an Fnr cysteine signature with three N-terminal Cys residues and a fourth in a β-roll (Green *et al.*, 2001). This feature is essential to the coordination of one [4Fe-4S] cluster per monomer, which enables the formation of the active, dimeric form of Fnr. Upon activation during anoxic conditions, Fnr dimers bind palindromic sequences called Fnr boxes (TTGAT-n_4-ATCAA) within the promoters of regulated genes, leading to activation or repression of transcription (Eiglmeier *et al.*, 1989). In the presence of oxygen, these [4Fe-4S] clusters undergo sequential oxidation, causing dissociation of the dimers and conformational changes within the monomers to release DNA (Crack *et al.*, 2004).

Initially thought only to repress genes for aerobic respiration and activate genes for anaerobic respiration, Fnr, like ArcA, affects the global cellular response to anaerobic conditions. Over 100 genes involved in a variety of cellular processes, many of which are unrelated to respiration, are regulated by Fnr (Kiley & Beinert, 2003). Fnr influences expression of genes involved in amino acid, fatty acid, and nucleic acid biosynthesis and metabolism, transcriptional and translational modification, the oxidative and acid stress responses, and transport of metal cofactors, peptides, and carbon sources. Virulence factors are also regulated by Fnr. It induces expression of the *Neisseria gonorrhoeae* major outer membrane protein antigen, the *Y. enterocolitica* phospholipase virulence factor, and numerous virulence genes in *S. enterica* serovar Typhimurium (Fink *et al.*, 2007; Householder *et al.*, 1999; Schmiel *et al.*, 2000).

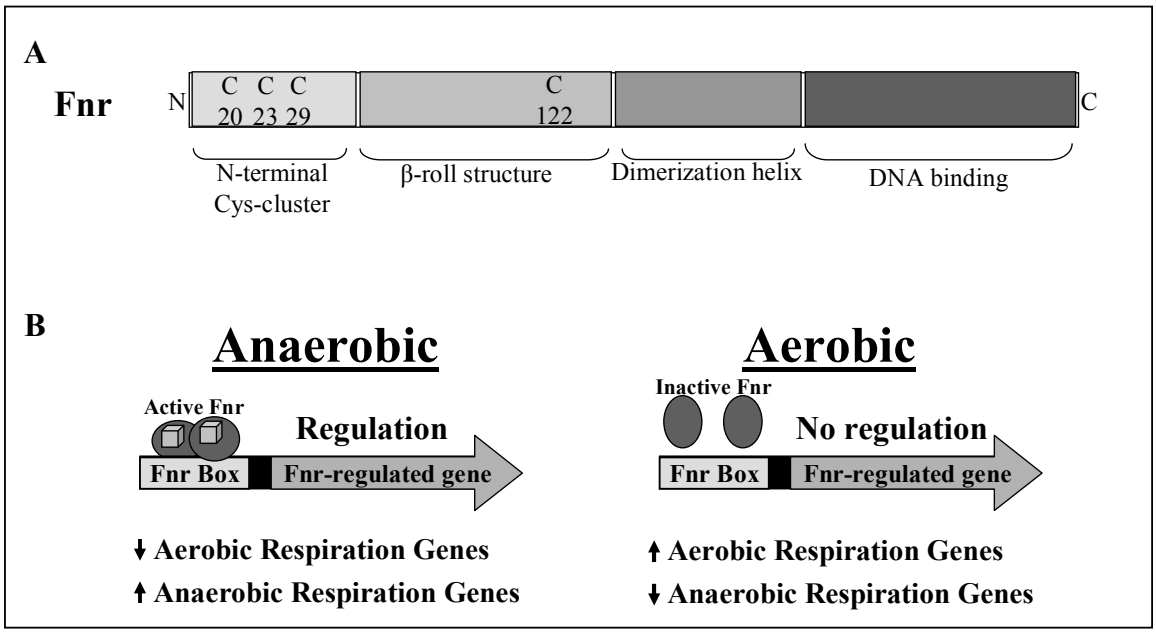


Figure 10. Fnr domains and regulation

A. Fnr is characterized in part by three N-terminal cysteine residues and a fourth found Cys within a β-roll motif. Fnr also has a dimerization domain that promotes homodimer formation when each monomer contains an iron-sulfur cluster, making C-terminal DNA binding domains competent for associating with DNA at binding sequences within promoters (Fnr boxes).

B. During anoxic conditions, the four Cys residues allow formation of a cubane [4Fe-4S] cluster (cubes) that enables active Fnr dimers to form. Fnr regulates target genes, such as those encoding enzymes involved in aerobic and anaerobic respiration. During aerobic conditions, oxygen disassembles the [4Fe-4S], causing Fnr to exist as inactive monomers.

3.3. REGULATION OF IRON ACQUISITION BY ANAEROBIOSIS

There are few reports in the literature indicating how the expression of iron transport genes changes in response to oxygen limitation. Analysis of the promoter sequence of the *feo* operon led to the identification of a region resembling the Fnr box, and a five-fold reduction in β-galactosidase activity from a *feo-lacZ* fusion was observed in the absence of *fnr*, indicating Fnr induces the expression of *feo* (Kammler *et al.*, 1993). In contrast to the anaerobic activation of *feo*, an anaerobic repression of *sitABCD* has been observed in *S. enterica* serovar Typhimurium (Ikeda *et al.*, 2005). This repression is independent of the anaerobic regulators ArcA or Fnr. Rather, the levels of the redox metals iron and manganese changes in response to oxygen availability, which affect the activity of Fur and MntR transcription factors to which they bind (Ikeda *et al.*, 2005). Thus, variations in the amounts of active Fur and MntR are responsible for the difference of *sit* expression in different oxygen concentrations. Additionally, the synthesis of aerobactin is known to be regulated by oxygen availability, as the activity of the first enzyme in the biosynthetic pathway, IucD mono-oxygenase, requires O_2 (Plattner *et al.*, 1989). Since aerobactin binds ferric iron, this regulation of IucD by oxygen availability allows aerobactin synthesis to be highest during the oxidative conditions in which its ligand is more abundant.

3.4. OXYGEN AVAILABILITY AND VIRULENCE

Studies with several pathogens have identified changes in virulence characteristics in response to low oxygen availability. Anaerobically grown *S. enterica* serovar Typhimurium showed significant increases in its ability to bind enterocytes, penetrate intestinal mucus, survive within spleen macrophages, and induce fluid accumulation in ligated rabbit ileal loops (Singh *et al.*, 2000). *S. enterica* serovar Typhi grown anaerobically demonstrated enhanced fluid accumulation in the ligated rabbit ileal loop (Kapoor *et al.*, 2002), while adhesion of enterohaemorrhagic *E. coli* (EHEC) to host cells increased following anaerobic growth (James & Keevil, 1999). Anaerobically induced metabolic pathways are also critical to some pathogens during infection. For example, *S. enterica* serovar Typhi and *Mycobacterium bovis* BCG mutants defective in anaerobic nitrate respiration are impaired in growth and persistence within host cells and tissues *in vivo* (Contreras *et al.*, 1997; Fritz *et al.*, 2002; Weber *et al.*, 2000). Anaerobic respiration in the presence of nitrate or TMAO increases the maturation of the EHEC TTSS and increases its secretion of effector proteins (Ando *et al.*, 2007), while the fumarate reductase is required for colonization of the stomach by *H. pylori* (Ge *et al.*, 2000). In addition, the loss of sugar fermentation genes, which are induced during anaerobiosis, causes a 2-3 log attenuation in mice and rat models of infection by *Neisseria meningitidis* serogroup B (Bartolini *et al.*, 2006). Collectively, these findings suggest anaerobic metabolism is required to enhance the growth and virulence of many human pathogens, and suggest that similar studies in *S. flexneri* would provide valuable insight into factors required for its pathogenesis.

4. Purpose of Investigation

Since iron acquisition is an important aspect of *S. flexneri* virulence and changes in redox affect aspects of transport and status of iron, it is important to determine whether genes involved in iron uptake are regulated in response to anaerobiosis and the primary anaerobic regulators, ArcA and Fnr. The expression of *S. flexneri* iron acquisition genes also varies greatly between *in vitro* and *in vivo* growth, and oxygen availability may play a role in this difference. Additionally, the site of *Shigella* infection of the human is anaerobic, and given the importance of ArcA and Fnr in the regulation of virulence of several pathogens, these transcription factors may also be involved in the pathogenesis of *S. flexneri*. Overall, this research will contribute to the current understanding of *S. flexneri* physiology and virulence and may ultimately shed light on our understanding of the eukaryotic environment.

II. MATERIALS AND METHODS

1. Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this study are described in Tables 1 and 2, respectively. The *E. coli* strain DH5α was used for the general cloning of recombinant DNA, while strain RM1602 (*dam*) was used in lieu of DH5α when restriction enzymes sensitive to DNA methylation were used. When generating mutants by conjugation, the DH5α(λ*pir*) strain was used as the donor strain with the helper strain MM294/pRK2013. Strain SA100 is a wild type *S. flexneri* (serotype 2a) clinical isolate from which all mutant strains were generated. SM100 is a spontaneous Str^r derivative of SA100. SA101, MBF100W, MBF200W, and MBF300W are spontaneous mutants that lost the ability to bind Congo red dye (Crb^-), indicative of a loss of virulence. The Crb^- mutants otherwise displayed phenotypes similar to parental strains in assays not addressing virulence properties.

Table 1. Strains used in this study

Strain	Relevant Characteristics	Source or reference
E. coli		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* [Φ80d*lac* Δ(*lacZ*)*M15*]	(Sambrook, 2001)
DH5α(*λpir*)	DH5α containing the λ*pir* prophage	(Sambrook, 2001)
ECL5331	MC4100 (wild type) *arcA*::*kan*	(Liu & De Wulf, 2004)
MM294/pRK2013	helper strain; for mobilization of R6K plasmids	R. Meyer, UT Austin
RM1602	F^- *dam-3*	R. Meyer, UT Austin
S. flexneri		
SA100	*S. flexneri* (serotype 2a) wild type	(Payne *et al.*, 1983)
SM100	SA100 Str^r	S. Seliger, UT Austin
SA101	SA100 Crb^-	(Daskaleros & Payne, 1987)
MBF100	SA100 *fnr*::*cam*	this study
MBF100W	SA100 Crb^- *fnr*::*cam*	this study
MBF200	SA100 *arcA*::*kan*	this study

MBF200W	SA100 Crb$^-$ *arcA*::*kan*	this study
MBF300	MBF200 *fnr*::*cam*	this study
MBF300W	SA100 Crb$^-$ *arcA*::*kan* *fnr*::*cam*	this study
SA190	SA100 *feoB*::*dhfr*	(Runyen-Janecky *et al.*, 2003)
SA240	SA100 *iucD*::Tn*5*	(Lawlor *et al.*, 1987)
SM166	SM100 *sitA*::*cam*	(Runyen-Janecky *et al.*, 2003)
SA192	SA190 *iucD*::Tn*5*	(Runyen-Janecky *et al.*, 2003)
SM191	SM166 *feoB*::*dhfr*	(Runyen-Janecky *et al.*, 2003)
SA167	SA240 *sitA*::*cam*	(Runyen-Janecky *et al.*, 2003)
SM193	SM191 *iucD*::Tn*5*	(Runyen-Janecky *et al.*, 2003)
SM1301	SM100 *fur*::*cam*	(Oglesby *et al.*, 2005)

Table 2. Plasmids used in this study

Plasmids	Characteristics	Source or reference
Gene Expression		
pCC1	Single- and inducible-copy expression vector	Epicentre Biotechnologies
pQE-2	IPTG-inducible gene expression vector	Qiagen
pWKS30	low-copy expression vector	(Wang & Kushner, 1991)
pMB*arcA*QE	pQE-2 with *arcA* coding region	This study
pMB*arcA*ccQE	pCC1 with IPTG-inducible *arcA* and *lacI* regions from pMB*arcA*QE	This study
pMB*fnr*WKS	pWKS30 with *fnr* coding region	This study
pMB*fur*QE	pQE-2 with *fur* coding region	This study
Mutant Construction		
pCVD442N	Sucrose-sensitive suicide vector with *Not*II restriction site in MCS; R6K ori	(Wyckoff *et al.*, 2006)
pDRIVE	PCR cloning vector	Qiagen
pDRIVE*fnr*	pDRIVE with SA100 *fnr* region	This study
pDRIVE*fnr*::*cam*	pDRIVE*fnr* with *cam* cassette disruption	This study
pMBcvd*fnr*::*cam*	pCVD442N with *fnr*::*cam* of pDRIVE*fnr*::*cam*	This study

pMTL*cam*	Source of *cam* cassette	(Wyckoff *et al.*, 1998)
Reporter Assays		
pLR29	*gfp* reporter vector pGTXN3 with RP4 mobility region of pGP704	(Runyen-Janecky & Payne, 2002)
pQF50	*lacZ* reporter vector	(Farinha & Kropinski, 1990)
pEG2	pLR29 with *sitA* promoter	(Runyen-Janecky & Payne, 2002)
pEG6	pLR29 with *iutA* promoter	L. Runyen-Janecky & E. Gonzales, UT Austin
pERM121	pQF50 with *shuA* promoter	E. Murphy, UT Austin
pMB*feoAlt*	pLR29 with altered *feoA* promoter	This study
pMB*feo*	pLR29 with *feoA* promoter	This study
pEG5	pLR29 with *fhuA* promoter	(Runyen-Janecky & Payne, 2002)
pMB*fhuA*Δ	pLR29 with 5' truncation of pEG5 promoter	This study
pMB*fhuA*Δ*alt*	pMB*fhuA*Δ with 5' base changes	This study
pHG*fhuA*Δ2	pMB*fhuA*Δ with 5' promoter truncation	M. Boulette and H. Graham
pMB*fhuA*Δ3	pMB*fhuA*Δ2 with 5' promoter truncation	This study
pMB*furAlt*	pLR29 with altered *fur* promoter	This study
pMB*fur*	pLR29 with *fur* promoter	This study

pMB*lld*	pLR29 with *lld* promoter	This study
pMB*minC*	pLR29 with *minC* promoter	This study
pMB*narG*	pLR29 with *narG* promoter	This study
pMB*ospC2*	pQF50 with *ospC2* promoter	This study
pMB*ospC3*	pQF50 with *ospC3* promoter	This study
pMB*shuA*	pLR29 with *shuA* promoter	This study
pMB*shuAalt*	pMB*shuA* with 5' base changes	This study
pMB*shuT*	pLR29 with *shuT* promoter	This study

2. Media, Reagents and Growth Conditions

Bacterial strains were stored at -70ºC in tryptic soy broth (TSB) + 20% glycerol. *E. coli* strains were grown in Luria Bertani broth (LB broth) or Luria Bertani agar (LB agar) (Gerhardt, 1994). *S. flexneri* strains were grown in RPMI [RPMI 1640 (Invitrogen Corporation) with L-glutamine without phenol red (Gibco; Invitrogen Corporation, Grand Island, NY), buffered with 100mM HEPES] supplemented with 2.5 μM $FeSO_4$ where indicated, or on tryptic soy broth agar plus 0.01% Congo red dye (Congo red agar) at 37ºC. Antibiotics were used at the following concentrations (per milliliter): 125 μg of carbenicillin (Carb), 200 μg of streptomycin (Str), 20 μg of kanamycin (Kan), and 7.5 μg of chloramphenicol (Cam). For anaerobic colony size assays, L broth agar was supplemented with 40mM $NaNO_3$, 5mM $NaNO_2$, 5% DMSO, 0.4% glucose, or 0.4% glycerol.

3. DNA Isolation

Plasmid DNA was isolated using QIAprep Spin Miniprep Kits (Qiagen, Santa Clarita, CA) according to the manufacturer directions. The isolation of DNA fragments from agarose gels was by QIAquick Gel Extraction Kits (Qiagen). DNA was eluted from columns in EB Buffer (10 mM Tris-Cl, pH 8.5) or double distilled H_2O, and stored at -20ºC.

4. Restriction Digestion and Ligation

Restriction digests and DNA ligations were performed following manufacturer's instructions. Restriction enzymes and molecular weight markers were purchased from New England Biolabs (Ipswich, MA) or Promega (Madison, WI). Molecular weight markers included λ DNA-*Hind*III digest and ΦX DNA-*Hae*III digest.

5. Transformation of Bacterial Strains

DNA from plasmid purifications or ligation reactions was introduced into $CaCl_2$-competent DH5α, DH5α(λ*pir*), or RM1602 by heat-shock transformation as described (Ausubel *et al.*, 1999). *S. flexneri* strains were made electrocompetent and transformed by electroporation as described (Conchas & Carniel, 1990).

6. Oligonucleotides

Oligonucleotide primers used for polymerase chain reaction (PCR) are listed in Table 3 and the appropriate sections below, and were designed using Clone Manager Suite 7 software (Scientific and Educational Software, Durham, NC). Real-time reverse

Table 3. Oligonucleotide primers

Primer	Sequence (5'-3')
Mutant Construction	
IWDArcAOut1	TATGTCATCTGCCACTGCCAGAGTC
IWDArcAOut2	CGAGCTTTATACGGTGCAGGAAGAG
MWFFnrDS1	ATACGGCTGTCGACTGGCGAGAT
FNRintFor	CGGCTTGAGCAGACCTATGA
FNRintRev	AACGTTACGCGTATGGCCAG
MWFnrUS1	CTGCAGAATGAGTGACGAGTTCAGTGGTGTCTG
Gene Expression	
MBarcAforQE	TTAAAGAGGAGAAATTAACTATGCAGACCCCGCACATTCTTATC
MBarcArevQE	GACGGTGGTAAAGCTTATTAATCTTCCAGATC
MWDfnrUS1	CTGCAGCATTACGGCCAACTGGCTGAACAA
MWDfnrDS1	GTCGACGTGATGAACCTTCTGCCAGATCAA
MBfurRevQE	TGATAAGCTTTGGCAGGAAATACGCAGTAATAAC
MBfurForQE	TTAAAGAGGAGAAATTAACTATGACTGATAACAATACCGCCCTA
Reporter Assays	
GFP2	CTGTTTCATATGATCTGGG
MBfeo5'arcA	CGGGATCCGTTAATATTTATCACTTC
MBfeo5'arcAalt	CGGGATCCGTTAATATCAGGGCGGACATTAACAACTGAAACC
EG17	GCTCTAGATGAATGGGGTCGCCGAG

EG14	CGGGATCCAGGCGGCGTATCTGACACTATG
MBfhuAarcA	TTTGGATCCGGTTGTTTATTAACCCTTC
MBfhuAarcAalt	TTTGGATCCGGTTGCCTATCCACCCTTCAGGAACGCTCAG
HGbox1	CGGATCCCGTACCGCTTGCGAACCC
HGbox2	CAGGATCCATCAGAGATATACCAATGGCGCGTTC
EG15	GCTCTAGAATCGGCGTATCGGTTTTAG
MBfurFor	CGGGATCCAATAAAATAATTGCTAC
MBfurAltFor	CGGGATCCAATAAAATAATTGCGTTTCGTTTGTAACTTTTGC
MBfurRev	GCTCTAGAGCGGAATCTGTCCTG
EG10	CGGGATCCAAAGCAGATAAGGCAGACAATCC
EG11	GCTCTAGAGGGCAGGGTCATAGTCAAAC
FZ057	GCCTCTTCGCTATTACGC
FZ072	GTTGAGGCCGTTGAGCAC
MBlldPrev	TACCGTTCTAGAGGCGGCGACGTATC
MBlldPfor	CTCCGGATCCACAATCTAAGGGCAATTC
MBminCfor	AAGCGGATCCTTCGCCGAGATATTTCCTTG
MBminCrev	TGTCTCTAGACGCCTGATGGATAACC
MBnarGfor	CTATGGATCCTGCGTAGTGATTACTTGGGC
MBnarGrev	CTGTTTCTAGAAGCGAAACCGGTCCAG
MBshuTA151	CAAGCGGATCCATTTTTGATTTTTATCAAGATATTTCG

MBshuTA151alt	CAAGCGGATCCATTTCCGACTTTTCTCGGGATATTTCGTCAGCTA
EG13	GCTCTAGAGCCAACAAACTCAAACGCAAC
MBshuATfor	CAGGATCCGCCAACAAACTCAAACGCAAC
MBshuATrev	TATCTAGAGTCAGTGGGGTAAAAAGAAACG
MBsp0066for	GCGTCTCTATTAGATCTATCATATTCTGAGC
MBsp0066rev	GATTTACTGCTTCAGGTATTTGGATCCAACATCCC
MBsp0122for	GGTTTCTGTAATAAGAGATCTGACATC
MBsp0122rev	GATTTACTGCTTCAGGTATTTGGATCCCC
Real-time RT-	
MBfurRT1	CATCACGTCAGTGCGGAAGA
MBfurRT2	GCGTCGTCAAACTGGTTCAGT
RrsA.for	CACGATTACTAGCGATTCCGACTT
RrsA.rev	CGTCGTAGTCCGGATTGGA
Probe Generation for EMSA	
pLR29EMSAfor	AGGACGCCCGCCATAAACTG
sitEMSArev	CCCAAGAGCATGGTTACTTTTTTTATTGAG
iucEMSAfor	GGGCGTATCTTCGATCCCTGGTGAC
feoArcRev	GAATAGTGAGTTACCCGGACTG
lldArcRev	CTGCCAATTGTGTTGTGTAGGG
furArcRev	CAACGGCAAGGCCACTTGAACAC

transcription-PCR (RT-PCR) oligonucleotide primers were designed by ABI Prism Primer Express Software (Applied Biosystems, Pleasanton, CA). Primers were manufactured by Integrated DNA Technologies (IDT, Coralville, IA). The lyophilized oligonucleotides were resuspended in double distilled water to a final concentration of 100 μM and stored at -20ºC.

7. DNA Sequencing

Cloned DNA constructs and PCR products were routinely sequenced using the automated dye termination procedure and analyzed on an ABI 377A DNA sequencer in the DNA Core Facility at The University of Texas Institute for Cellular and Molecular Biology (Austin, TX).

8. DNA Amplification

PCR to amplify DNA templates was carried out using a GeneAmp PCR System 2400 (Applied Biosystems) or PTC-200 Peltier Thermal Cycler (MJ Research, Inc., Watertown, MA). PCR was routinely performed using *Taq* polymerase (Qiagen), or *Pfu* polymerase for higher fidelity when cloning large DNA sequences (Stratagene Cloning

Systems, La Jolla, CA). Unless otherwise specified, PCR reactions contained template DNA, 200 μM of each dNTP, 2 μM each primer, 1X concentration reaction buffer (supplied by manufacturer with each polymerase), and 1-5 units enzyme in a reaction volume of 50–100 μl. For DNA amplification using Taq polymerase, the standard thermocycler program included an initial template dissociation step for 1 minute at 94ºC, followed by 30 cycles of a 45-second dissociation step at 94ºC, 45-second annealing step at appropriate temperature for the primer pair (usually 50-55ºC), and a 72ºC elongation step for the appropriate amount of time to synthesize a product of the desired length (1 minute for each 1000 base pairs of expected product). A final extension of 5 minutes at 72ºC finished the reaction, after which the thermocycler was programmed to remain indefinitely at 4ºC. The thermocycler program was similar for DNA amplification with *Pfu* polymerase with the exception of extended elongation times (1 minute for each 500 base pairs of expected product).

9. Construction of Recombinant Plasmids and Strains

9.1. Construction of Mutant Strains

Mutant strains used are listed in Table 1. The *S. flexneri arcA::kan* mutant was constructed by bacteriophage P1 transduction as previously described (Sambrook, 2001).

P1 lysates were made following growth of P1 vir on the *E. coli arcA::kan* mutant, ECL5331 (Liu & De Wulf, 2004). Insertional inactivation of the *arcA* gene in MBF200 was confirmed by PCR using primers flanking the *arcA* gene, IWDArcAOut1 and IWDArcAOut2.

An *S. flexneri fnr* mutant was generated by allelic exchange. The SA100 genomic region including *fnr* and surrounding sequence was amplified by PCR using MWDFnrUS1 and MWDFnrDS1 primers, and the pDRIVE vector and PCR product were digested with *Pst*I and Sail and ligated. A *cam* cassette was excised from pMTL*cam* using *Sma*I and ligated into pDRIVE*fnr* digested with the *Bcl*I and made blunt with the Klenow fragment of DNA polymerase I (New England Biolabs (NEB), Ipswich, MA). *fnr*::*cam* was then excised from pDRIVE*fnr*::*cam* using *Sma*I and ligated into *Sma*I-digested pCVD442N. The resulting plasmid, pMBcvd*fnr*::*cam*, was then mated into SM100 by tri-parental conjugation. Primary integrants were selected by growth in the presence of Carb, Str, and Cam, and verified using primers MWFFnrDS1 and FNRintFor. The *fnr*::*cam* mutant was isolated by growth in the presence of sucrose and Cam, and was confirmed by PCR using primer pairs FNRintFor and FNRintRev, and MWFnrUS1 and MWFFnrDS1. MBF100 (SA100 *fnr::cam*) and MBF300 (MBF200 *fnr::cam*) were obtained by P1 transduction of *fnr::cam* from SM100 *fnr::cam* to SA100 and MBF200, respectively. PCR using the primer pairs MWFFnrDS1 and MWFnrUS1 was again used to verify *fnr* gene disruption, and primers IWDArcAOut1 and IWDArcAOut2 were used to verify the *arcA* gene disruption.

MBF100W, MBF200W, and MBF300W were spontaneous mutants that lost the ability to bind Congo red dye (Crb^-), indicative of loss of virulence. The Crb^- mutants

were used for procedures in which maintaining virulence of the strains posed unnecessary risk.

9.2. CONSTRUCTION OF EXPRESSION PLASMIDS

9.2.1. ArcA Expression

For complementation of the *arcA* mutation, a single-copy, IPTG-inducible *arcA* vector was engineered by first cloning the *arcA* gene under the inducible T5 promoter of the pQE-2 vector (Qiagen). SA100 genomic DNA was used as template for PCR using the primers MBarcAforQE and MBarcArevQE. The PCR product and pQE-2 vector were digested with *Bse*RI and *Hind*III and ligated to make pMB*arcA*QE. The *Fsp*I fragment of pMB*arcA*QE, including the *lacI* and *P*T5-*arcA* genes, was excised from this construct and ligated into the blunt-cloning ready pCC1 vector (Epicentre Biotechnologies, Madison, WI). The resulting plasmid, pMB*arcA*ccQE, was verified by DNA sequencing.

9.2.2. Fnr Expression

Complementation of *fnr* mutation was performed using pMB*fnr*WKS, which contained the *fnr* gene expressed from its own promoter in the low-copy pWKS30 vector. The *fnr* gene and surrounding sequence of SA100 genomic DNA was amplified by PCR

using primers MWDfnrUS1, which incorporated a *Pst*I restriction enzyme site 5' of the *fnr* promoter region, and MWDfnrDS1, which incorporated as *Sal*I restriction enzyme site 3' of the *fnr* coding region. The product and vector were digested with *Pst*I and *Sal*I and ligated, and pMB*fnr*WKS was confirmed by DNA sequencing of the inserted *fnr* gene.

9.2.3. Fur Expression

For the over-expression of *fur*, a plasmid containing *fur* under the control of an IPTG-inducible promoter, pMB*fur*QE, was constructed. MBfurForQE and MBfurRevQE primers were used in PCR to amplify SA100 *fur* with engineered 5' *Bse*RI and 3' *Hind*III sites. The PCR product and pQE-2 vector were digested with *Bse*RI and *Hind*III and ligated. Transformants were screened by plasmid purification, restriction digestion, and electrophoresis, and the resulting pMB*fur*QE plasmid was confirmed by DNA sequence analysis.

10. Tissue Culture and Plaque Assays

Henle cells (Intestine 407 cells; American Type Culture Collection, Manassas, VA) were routinely grown to confluency and passaged in either Minimum Essential

Medium (MEM) or RPMI 1640 (Invitrogen Corporation) medium supplemented with 10% Bacto Tryptose Phosphate Broth (Difco; Becton, Dickinson and Company, Sparks, MD), 2 mM glutamine, MEM Non-essential Amino Acid Solution (Invitrogen Corporation), and 10% Fetal Bovine Serum (Invitrogen Corporation) in a 5% CO_2 atmosphere at 37°C. Aerobic plaque assays were performed as described previously (Runyen-Janecky *et al.*, 2003), except that the medium was supplemented with 100mM HEPES (pH7.5), agarose was omitted from the overlay, and medium overlay was aspirated after 48 hours of incubation to stain plaques with Wrights-Giemsa differential stain. Anaerobic plaque assays were performed identically to the aerobic plaque assays, except that 6-well plates were incubated in BD BBL GasPack Pouch Anaerobic System (Becton, Dickinson, and Company) for the duration of the experiment. The assay was performed under microaerophilic conditions as well, using BBL Campy Pouch Microaerophilic System (Becton, Dickinson, and Company).

11. Microarray Analysis

11.1. CONSTRUCTION OF MICROARRAYS

Microarrays were constructed and post-processed as described previously (Oglesby *et al.*, 2005). Briefly, hybrid *E. coli*/*Shigella* microarrays were designed using

commercially-available 70-mer oligonucleotides (Qiagen) for the *E. coli* K-12 and EHEC sequenced genomes and custom-designed 70-mer oligonucleotides corresponding to *Shigella*-specific genes and putative sRNA sequences. Poly-L-lysine coated glass microscope slides were prepared (detailed at www.microarrays.org) and imprinted with oligonucleotide spots using a robotic arrayer and Array Maker 2.4 as described by MGuide (chipmunk.icmb.utexas.edu/ilcrc/protocols/index.shtml). Microarrays were then post-processed as described by MGuide prior to hybridization.

11.2. ISOLATION OF RNA AND GENERATION OF CDNA

Wild type, MBF100, MBF200, and MBF300 *S. flexneri* batch cultures were grown aerobically in RPMI with or without added iron (as indicated) to mid-logarithmic phase aerobically in a BIOFLO 110 Fermenter/Bioreactor (New Brunswick Scientific, Edison, NJ) to maintain constant pH, dissolved oxygen concentration, temperature, and agitation. The aerobically grown bacteria, sparged continuously with air, were harvested during mid-logarithmic growth. The dissolved oxygen was then depleted by switching the sparging source from air to nitrogen gas. Fifteen minutes after oxygen depletion, the anaerobic bacteria were isolated. All bacterial samples were added to one-fifth volume 95% ethanol: 5% phenol solution to prevent further changes in the transcriptional profile and refrigerated until RNA isolations were performed.

RNA was isolated from bacterial cultures using RNeasy Midi Kits (Qiagen). Reverse transcription of RNA was performed using 5µg of pd(N)6 randomized hexamer

primers (Amersham Biosciences, Piscataway, NJ) with 15 µg total RNA for 10 minutes at 65ºC. Primed RNA was incubated with 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.2 mM dTTP, 0.3 mM amino allyl modified dUTP (Sigma-Aldrich, St. Louis, MO), 0.01 M dithiothreitol (DTT), 120 units RNaseIn (Promega, Madison, WI), 800 units SuperScript II Reverse Transcriptase (Invitrogen Corporation), and 1X SuperScript RT Buffer at 42° for 1 hour, after which an additional 400 units of SuperScript II were added and the reaction was incubated for an additional hour. NaOH was added to a final concentration of 50 mM to hydrolyze RNA. After incubation at 65ºC for 10 minutes, the reactions were neutralized by the addition of HCl to a final concentration of 50 mM. The reactions were then concentrated to 9µl using a Microcon-30 column (Millipore, Billerica, MA).

11.3. LABELING AND HYBRIDIZATION

The concentrated amino allyl-dUTP-incorporated cDNA was coupled to reactive Cy3 or Cy5 dyes (Amersham Biosciences) by buffering each sample with 1 µl of 10 M sodium bicarbonate (pH 10) and combining with either Cy3 or Cy5 reactive dye. cDNA from strains grown aerobically was routinely labeled with Cy3 and cDNA from strains grown anaerobically was labeled with Cy5 in arrays comparing aerobic and anaerobic gene expression. For the arrays comparing anaerobic growth of the wild type organism to that of a mutant, the wild type cDNA was labeled with the Cy3, while the Cy5 dye was used to label the mutant cDNA. The coupling reactions were incubated in the dark for 1 hour, and uncoupled dye was removed by purification of labeled samples on MinElute

Spin columns (Qiagen). Cy3- and Cy5-labeled samples were combined with 3X SSC and 0.25% SDS to make the hybridization probe. The probe was carefully added to the microarray beneath a coverslip and sealed in a hybridization chamber. cDNA probes were hybridized to arrays for 3 hours at 65ºC, and then washed as described at chipmunk.icmb.utexas.edu/ilcrc/protocols/index.shtml.

11.4. ANALYSIS OF MICROARRAY DATA

Microarrays were scanned using the Genepix Array Scanner 4000A (Axon Instruments, Union City, CA). Preliminary analysis of microarrays was performed using the Genepix 5.0 Software, and normalization of microarray data was carried out by the Longhorn Array Database (powered by the Stanford Microarray Database). Normalized data were filtered so that spots with a regression correlation lower than 0.6 and those that were in areas of high background were excluded. Additionally, genes that did not exhibit greater than a two-fold difference in expression in at least two arrays and those that showed inconsistent patterns of induction or repression were excluded from further analysis. Fold-changes were determined by averaging those values that met the criteria listed above, and standard deviations were reported when greater than two data sets passed these quality controls. Images were generated using Cluster 3.0 (Stanford University) and Cluster TreeView (Eisen Software).

12. GFP-Reporter Assays

12.1. Construction of GFP-Reporter Plasmids

Plasmids for GFP-reporter assays (Table 2) were constructed by PCR amplification of the promoter of each gene and ligation into the promoterless *gfp* vector pLR29. Genomic DNA from wild type *S. flexneri* or *S. dysenteriae* (*shu* fusions only) was used as template with oligonucleotide primers in the combinations listed in Table 4. The 5' primers contained *Bam*HI restriction sites while the 3' primers contained Bay sites, and after PCR, promoter DNA and pLR29 vector DNA were double-digested with these restriction enzymes. The insert and vector DNA were ligated using T4 DNA ligase, and 10 µl ligation reactions were used for transformation into DH5α. Transformations were plated onto LB agar with Carb, and plasmid DNA from transformants was isolated and screened by PCR. All plasmids were sequenced using the primer GFP2 to determine that the correct promoter sequences were inserted upstream of the promoterless *gfp* gene. Plasmids were then electroporated into SA101, MBF100W, MBF200W, and MBF300W to assay promoter activity.

Table 4. Primer combinations used to amplify promoters for reporter fusions

Plasmids	5' Oligonucleotide Primer	3' Oligonucleotide Primer
GFP-reporter fusions		
pMB*feoAlt*	MBfeo5'arcAalt	EG17
pMB*feo*	MBfeo5'arcA	EG17
pMB*fhuA*Δ	MBfhuAarcA	EG15
pMB*fhuA*Δ*alt*	MBfhuAarcAalt	EG15
pHG*fhuA*Δ2	HGbox1	EG15
pMB*fhuA*Δ3	HGbox2	EG15
pMB*furAlt*	MBfurAaltFor	MBfurRev
pMB*fur*	MBfurAFor	MBfurRev
pMB*lld*	MBlldPfor	MBlldPrev
pMB*minC*	MBminCfor	MBminCrev
pMB*narG*	MBnarGfor	MBnarGrev
pMB*shuA*	MBshuTA151	EG13
pMB*shuAalt*	MBshuTA151alt	EG13
pMB*shuT*	MBshuATfor	MBshuATrev
β-galactosidase reporter fusions		
pMB*ospC2*	MB0066for	MB0066rev
pMB*ospC3*	MB0122for	MB0122rev

12.2. GROWTH CONDITIONS AND FLUORESCENCE DETERMINATION

For the GFP reporter assays, Crb^- strains containing the desired plasmids were plated from freezer stocks onto Congo red agar supplemented with appropriate antibiotics and grown overnight at 37°. The following day, three isolated colonies were used to start three separate overnight cultures in 2ml RPMI with 2.5 μM $FeSO_4$, and appropriate antibiotics, and the cultures were grown overnight at 37°. These overnight cultures were diluted to an OD_{600} of 0.05 into 2ml RPMI with 100mM HEPES and Carb for plasmid maintenance and grown aerobically at 37° for 2 hours before sub-culturing to an OD_{600} of 0.03 into the same medium. The cultures were grown for 2 hours either aerobically with vigorous shaking or anaerobically in Oxoid AnaeroJars with AnaeroGen sachets and Anaerobic Indicators (Oxoid Ltd., Hampshire, England), as indicated. Each culture was diluted in cuvettes to obtain 1 ml at OD_{600}~0.08. After recording the OD_{600} of each culture, the relative fluorescence of the culture was read in the VersaFluor fluorometer (Bio-Rad Laboratories, Hercules, CA). The instrument was blanked using the strains with the pLR29 negative control and the range was set to 15,000 Relative Fluorescent Units (RFU) using the pMB*minC* vector, which expresses *gfp* constitutively. The data are shown as relative expression, with the culture giving maximal expression set to 100%. The average RFU/OD_{600} of each transcriptional fusion was normalized to the RFU/OD_{600} of the pMB*minC* vector in the same strain. Results are the average of three independent experiments, with error bars indicating one standard deviation. The plasmids pMB*narG* and pMB*lld* served as positive controls for Fnr and ArcA regulation, respectively.

13. β-galactosidase-Reporter Assays

13.1. Construction of β-galactosidase-Reporter Plasmids

Plasmids for β-galactosidase-reporter assays (Table 2) were constructed by PCR amplification of the promoter of each gene and ligation into the promoterless *lacZ* vector pQF50. SA100 DNA was used as template with oligonucleotide primers in the combinations listed in Table 4. The 5' primers contained *Bgl*II restriction sites while the 3' primers contained *Bam*HI sites, and after PCR, promoter DNA and pQF50 vector DNA were digested with these restriction enzymes. The insert and vector DNA were ligated using T4 DNA ligase, and 10 µl ligation reactions were used for transformation into DH5α. Transformations were plated onto LB agar with Carb, and plasmid DNA from transformants was isolated and screened by PCR. All plasmids were sequenced using the primers FZ057 and FZ072 (Z. Feng) to determine that the correct promoter sequences were inserted upstream of the promoterless *gfp* gene. Plasmids were then electroporated into SA100 to assay promoter activity.

13.2. Growth Conditions and Determination of b-galactosidase Activity

For the β-galactosidase reporter assays, strains were grown as for the GFP-reporter assays. Every hour of growth for 6 hours and again at 24 hours, the OD_{600} and

β-galactosidase activity were measured as described (Miller, 1972). The plasmids pQF50 and pERM121 served as controls.

14. ArcA Weight Matrix and Sequence Motif

Sequences of the promoters for which ArcA binding has been demonstrated were entered into the SeSiMCMC interface (http://favorov.imb.ac.ru/SeSiMCMC), and a conserved weight matrix for ArcA sequence recognition was generated based on the algorithm used (Favorov *et al.*, 2005). The promoters included in the search were those of *E. coli aldA*, *cydA*, *focA-pfl*, *glcC*, *gltA-sdh*, *icdA*, *lld*, *lpdA*, *moe*, *ptsG*, *sodA*, and *S. flexneri feoA*, *fur*, and *iucA*. The sequence logo was then obtained by inputting the weighted matrix derived from the multiple sequence alignment into the interface at http://weblogo.berkeley.edu.

15. Immunoblot Assays

S. flexneri MBF200/pMB*arcA*ccQE, SA100/pCC1, and MBF200/pCC1 were grown in the presence of dilutions of IPTG to estimate the amount of IPTG necessary to induce wild-type levels of ArcA in MBF200/pMB*arcA*ccQE. Cultures were grown in the

same manner as for real time RT-PCR assays, except that 10-fold serial dilutions of IPTG were added to the final sub-culture medium. The optical density was measured at 600nm and equivalent cell numbers were harvested. Cells were washed in Tris-buffered saline (pH7.4) and resuspended to 100µl, to which 20 µl 6X Laemmli buffer was added. Samples were boiled for five minutes before loading equal volumes on a 15% polyacrylamide gel. The protein gel was electrophoresed, transferred, and blotted as described (Ausubel *et al.*, 1999). The blot was incubated with a 1/5000 dilution of goat α-ArcA primary antiserum (generously provided by P. Silverman (Silverman *et al.*, 1991)) followed by rabbit α-goat horseradish peroxidase (HRP)-conjugated secondary antibody, and detected by chemiluminescence.

16. Real Time RT-PCR

For bacterial isolation for real time RT-PCR, SA100/pCC1, MBF200/pCC1, and MBF200/pMB*arcA*ccQE strains were grown as described for GFP-Reporter Assays (section II.12.2), with two notable exceptions. Since the Crb^+ strains used in this assay grow more slowly than Crb^-, the cultures were grown for three hours instead of the two hours indicated for the Crb^- strains used in the GFP reporter assays. Additionally, 1µM IPTG was added to the final sub-culture medium to induce ArcA expression. This concentration of IPTG was shown by α-ArcA (generously provided by P. Silverman (Silverman *et al.*, 1991)) immunoblots to induce wild-type levels of ArcA. One-fifth volume of 95% ethanol: 5% phenol (v:v) was added to logarithmically growing anaerobic

cultures and RNA was isolated on RNeasy Mini Columns (Qiagen). RNA was DNase-treated on column (Qiagen DNase I) and again after elution using Amplification Grade DNaseI (Invitrogen Corporation) according to the manufacturers' instructions. cDNA was generated from approximately 5 µg of each RNA sample using the High Capacity cDNA Archive Kit (Applied Biosystems). RT-PCR reactions contained 1X Power SYBR Green PCR Master Mix (Applied Biosystems), 800 nM of forward and reverse primers (IDT), and $1/200^{th}$ of the cDNA reaction in a total volume of 25 µl. *fur* cDNA was detected using the primers MBfurRT1 and MBfurRT2. *rrsA* cDNA was detected using the primers RrsA.for and RrsA.rev. Real Time RT-PCR was carried out in an Applied Biosystems 7300 Real Time PCR System, and analysis was performed using the 7300 Real Time PCR System Software. Standard curves for each primer set were generated using cDNA obtained from 10-fold dilutions of SA100 RNA, and the amount of cDNA in each sample was extrapolated from the standard curve. The relative amounts of *fur* experimental cDNA were normalized by dividing the values by the relative amounts of *rrsA* control cDNA in each sample.

17. Antibody Super-shift Assays

17.1. Probe Generation and Labeling

To determine ArcA binding of promoter sequences, the promoter-*gfp* fusion plasmids (Table 2) served as templates for PCR reactions to generate probes. The pLR29EMSAfor primer was used with the reverse primers lldArcRev (*lld* probe), feoArcRev (*feo* and *feoAlt* probes), furArcRev (*fur* and *furAlt* probes), or sitEMSArev (*sit* probe). The *iuc* probe was generated using iucEMSAfor and EG11 primers.

DNA fragments of the correct sizes were extracted from gels and digested with *Xma*I (probes generated with the pLR29EMSAfor 5' primer) or *Xba*I (*iuc* probe). The probes were gel purified and the ends filled in using Klenow fragment (NEB) and a mixture of nucleotides for cold probes, replacing dCTP with [α-^{32}P]dCTP (Perkin Elmer, Boston, MA) for radio-labeled probes. Unincorporated nucleotides were removed using Micro Bio-Spin P-30 Tris Chromatography Columns (Bio-Rad Laboratories), and all probes were phenol:chloroform extracted and ethanol precipitated (Ausubel *et al.*, 1999). Radioactivity was measured by scintillation counting. The probes were quantified by determining the A_{260}/A_{280} and the concentrations were adjusted to equivalent molar ratios.

17.2. PREPARATION OF CELL EXTRACTS

Cell extracts from MBF200/pCC1 and MBF200/pMB*arcA*ccQE were prepared as previously reported for *E. coli arcA* and *arcA*-overexpressing strains (Tardat & Touati, 1993), with the exception of growth conditions. Overnight cultures were grown in HEPES-buffered RPMI with 2.5 µM $FeSO_4$, Cam, and Kan, and sub-cultured 1/100 into

medium without added iron. Cultures were grown aerobically at 37° to mid-logarithmic phase, then diluted into the same medium with 1μM IPTG and grown for 2 hours under anaerobic conditions.

17.3. ELECTROPHORETIC MOBILITY SHIFT AND PHOSPHORESCENCE ANALYSIS

The antibody super-shift assays were performed essentially as described (Ausubel *et al.*, 1999). Reactions contained binding buffer (10 mM Tris-Cl (pH 7.4), 10% glycerol, 10 mM $CaCl_2$, 100 mM KCl, 1 mM EDTA, 5 μg/ml BSA, 1 mM DTT, and 1 μg poly (dIdC)), 5 μg of crude protein extract, 1 μl of antiserum diluted 1/1000, and approximately 1 ng labeled probe in 30 μl. Cold competitor DNA probes were added to the reaction mixture at 2X, 4X, and 8X molar excess of the radio-labeled probe. Radioactive bands separated by electrophoresis in a 5% polyacrylamide/TBE gel were visualized with a Bio-Rad Molecular Imager FX after overnight exposure of the dried gel to a phosphor screen (Bio-Rad Laboratories). Fluorophore band intensity was analyzed using Quantity One software (Bio-Rad Laboratories).

III. RESULTS

1. *S. flexneri* Gene Expression in Response to Anaerobiosis

To determine the global transcriptional response of *S. flexneri* to anaerobiosis, microarray analysis was performed. Microarray technology has been used to determine the pattern of gene regulation of aerobically and anaerobically grown *E. coli* K-12 (Salmon *et al.*, 2003; Salmon *et al.*, 2005; Soupene *et al.*, 2003). Anaerobic expression profiles have also been determined comparing *E. coli* K-12 wild type and *fnr* deletion strains (Kang *et al.*, 2005; Khullar *et al.*, 2003), as well as comparing wild type with *arcA* mutants (Liu & De Wulf, 2004; Salmon *et al.*, 2005). These global transcriptional approaches have further defined the ArcA and Fnr regulons and the metabolic changes that occur in response to oxygen availability.

S. flexneri was grown under aerobic conditions in defined medium and compared to cultures undergoing the transition to anaerobiosis. These arrays were performed in the presence and absence of $FeSO_4$. Aerobic cultures were sampled once mid-logarithmic growth was reached. The air was then switched to nitrogen gas, and anaerobic cultures were isolated fifteen minutes after oxygen depletion. Although we expect many similarities between the *S. flexneri* and *E. coli* data, some differences are also likely. In *S. flexneri* many genes responsible for adaptation to anaerobiosis and anaerobic

metabolism in *E. coli* are predicted to be pseudogenes (Jin *et al.*, 2002; Wei *et al.*, 2003). The *S. flexneri* genome also contains a large number of genes for virulence, including some involved in iron acquisition, that are not present in the *E. coli* K-12 strains studied by microarray analyses. Additionally, our arrays are designed to compare the transition to anaerobiosis in otherwise identical cultures, whereas the *E. coli* arrays were performed with separate cultures grown during either aerobiosis or anaerobiosis. Differences in culture medium are also predicted to impact the microarray results, since the *S. flexneri* cultures are grown in rich, defined medium used for tissue culture assays, while the *E. coli* cultures were grown in either LB with glucose or minimal defined medium.

For profiling the transcriptional response to anaerobiosis in *S. flexneri*, wild type SA100 was grown in batch cultures in a chemostat to control the pH and oxygenation during growth. This ensures that the changes of gene expression detected in the microarrays can be attributed solely to the transition to anaerobiosis. Throughout growth, the pH of the medium was maintained at 7.5 +/- 0.1 by addition of 2M HEPES of pH 7.2 or 7.8, depending on pH measured by a probe submerged in the culture vessel. Culture agitation and continuous air flow were also maintained to keep the dissolved oxygen concentration maximal during aerobic growth. Since samples could be taken from the chemostat culture without changing the growth conditions inside the vessel, the OD_{600} was determined at regular intervals to follow the growth the culture. Additionally, to prevent transcriptional changes due to exposure of anaerobic samples to aerobic conditions before RNA isolation, cultures were collected in one fifth volume phenol:ethanol. These precautionary measures were taken to generate microarray data that could accurately and consistently reflect differences between anaerobic and aerobic conditions.

1.1. GENES REGULATED BY ANAEROBIOSIS IN THE PRESENCE OF IRON

S. flexneri was grown in the presence of iron, and genes exhibiting at least a two-fold change in mRNA levels between aerobiosis and after the transition to anaerobiosis in at least two arrays were analyzed (Table 5 and Appendix A). These data demonstrate that proteins involved in diverse cellular processes were regulated by oxygen availability in *S. flexneri*. Many genes previously reported to be regulated by anaerobiosis or by the anaerobic transcription factors ArcA or Fnr in *E. coli* showed the expected regulation in the *S. flexneri* transcriptional profiles (Table 5 and Appendix A). Examples include metabolic genes involved in aerobic (*nuo*) and anaerobic respiration (*frd*, *hyp*, *hyb*, *mod*, *nap*, *nar*, and *nir*), glycolysis (*gpmA*), pyruvate metabolism (*lpd-ace*), and the TCA cycle (*icd*, *mdh*, *sdh*, and *suc*) (Ikeda *et al.*, 2005; Kang *et al.*, 2005; Khullar *et al.*, 2003; Liu & De Wulf, 2004). Several stress response genes known to be regulated by oxygen in *E. coli* were regulated similarly in *S. flexneri.* These include aerobically elevated oxidative stress genes (*isc*, *sod*, *sox*, and *tpx*), and the anaerobically elevated acid responsive gene *hdeA* (Table 5, Appendix A). Additionally, a number of other loci involved in sugar and amino acid transport and metabolism, fatty acid biosynthesis, and protein translation, as well as genes encoding numerous proteins of unknown function, were regulated by oxygen availability (Appendix A). Since many genes that were regulated in *E. coli* were also regulated by oxygen availability in *S. flexneri*, the results provided confirmation that the experimental design was appropriate to determine the transcriptional differences between growth in the presence and absence of oxygen in *S. flexneri*. The arrays also demonstrated that many of the global metabolic changes that occur in *E. coli* in response to anaerobiosis also take place in *S. flexneri*.

Table 5. Microarrays demonstrate transcriptional changes of selected genes in the presence of iron in response to oxygen availability in *S. flexneri*

Gene	Function	Fold-change [a] Range	Average	S.D.
Transcripts elevated aerobically				
Metabolic genes				
nuoI	NADH dehydrogenase	1.3-2.6	2.0	0.7
nuoE	NADH dehydrogenase	2.0-2.9	2.4	0.5
nuoC	NADH dehydrogenase	2.1-2.2	2.2[b]	
gpmA	Glycolysis	2.7-5.1	3.5	1.4
lpdA	Lipoamide dehydrogenase	9.3-11.5	10.4[b]	
aceE	Pyruvate dehydrogenase	9.0-10.5	9.7[b]	
icdA	Isocitrate dehydrogenase	2.4-10.4	6.4[b]	
mdh	Malate dehydrogenase	5.7-13.9	9.7	4.1
sdhD	Succinate dehydrogenase	3.9-4.7	4.3[b]	
sdhB	Succinate dehydrogenase	2.9-3.9	3.4[b]	
sucA	Succinyl transferase	2.5-3.1	2.8[b]	
sucC	Succinyl transferase	7.7-7.8	7.8[b]	
sucD	Succinyl transferase	3.6-7.9	5.7[b]	
Stress response genes				
yfhJ	Fe-S cluster formation	1.3-2.5	2.1	0.6

iscA	Fe-S cluster formation	1.9-5.8	3.4	2.1
iscU	Fe-S cluster formation	4.0-7.0	5.5	
iscS	Fe-S cluster formation	4.6-6.5	5.6	
sodA	Superoxide dismutase	3.2-11.9	7.6[b]	
sodB	Superoxide dismutase	1.9-4.6	2.8	1.5
soxS	Oxidative stress transcription factor	1.9-4.6	2.9	1.5
tpx	Thiol peroxidase	6.6-8.2	7.4[b]	
Virulence plasmid genes				
mxiI	TTSS apparatus	1.4-2.4	2.0	0.5
mxiD	TTSS apparatus	2.1-2.5	2.3[b]	
Transcripts elevated anaerobically				
Metabolic genes				
frdA	Fumarate reductase	1.0-4.3	2.5	1.7
hypA	Hydrogenase maturation	1.5-6.4	3.5	2.6
hypB	Hydrogenase maturation	1.6-10.3	5.2	4.6
hypD	Hydrogenase maturation	3.3-4.4	3.8	0.6
hypE	Hydrogenase maturation	1.7-3.7	2.5	1.1
hybD	Hydrogenase synthesis	1.0-2.4	1.9	0.8
hybB	Hydrogenase synthesis	1.1-7.5	4.8	3.3
modA	Molybdate transport	7.7-9.5	8.6[b]	
modB	Molybdate transport	5.0-8.1	6.6[b]	
napG	Nitrate reduction	1.8-4.3	3.1	1.3

narK	Nitrate reduction	3.4-5.3	4.3[b]	
narG	Nitrate reduction	9.2-39.2	27.8	16.2
narH	Nitrate reduction	4.0-18.4	13.2	8.0
narJ	Nitrate reduction	2.2-18.1	10.2[b]	
narI	Nitrate reduction	3.7-14.5	9.1[b]	
nirB	Nitrite reduction	5.4-57.0	31.2[b]	
Stress response gene				
hdeA	Acid stress chaperone	2.9-3.8	3.3	0.5

[a]Fold-change and standard deviation (S.D.) were calculated as described in Materials and Methods.
[b]Standard deviations were not available if fewer than three data sets met quality controls.

There were few differences in *S. flexneri* virulence gene expression patterns between aerobic and anaerobic conditions. *mxiD* and *mxiI* transcripts were elevated roughly two-fold aerobically, although the expression of other *mxi-spa* genes in the same operon was not affected. Whether additional variables needed to be taken into account to observe transcriptional changes in virulence genes in response to oxygen availability, for example the presence of an additional metabolite in the medium or assaying a different phase of bacterial growth, or whether these genes simply are not regulated by oxygen is not clear.

1.2. GENES REGULATED BY ANAEROBIOSIS IN THE ABSENCE OF IRON

Arrays were performed comparing the transition from aerobic to anoxic conditions in the absence of iron since ferri-Fur repression of iron-responsive genes could inhibit aerobic and anaerobic transcriptional differences of these genes from being observed. Similar to the *S. flexneri* arrays comparing aerobic and anaerobic differences in the presence of iron, many of these genes are also regulated in *E. coli* by oxygen availability or by ArcA or Fnr (Ikeda *et al.*, 2005; Kang *et al.*, 2005; Khullar *et al.*, 2003; Liu & De Wulf, 2004). Some of the genes that were induced aerobically in *E. coli,* as well as in *S. flexneri* in either iron abundance or restriction, included TCA cycle loci *acnA* and *mdh*, the *lpdA-aceE* operon for the pyruvate dehydrogenase complex, and the oxidative stress response genes *iscAUR*, *sodA*, and *tpx* (Table 6 and Appendix B). Additionally, the *ndk* and *nrd* genes encoding the aerobic ribonucleotide reductases, the F_1F_0 ATPase *atpC-F* operon, *sufESDCBA* and *zwf* genes involved in the response to oxidative stress were elevated aerobically in *E. coli* or aerobically during iron limitation

Table 6. Microarrays demonstrate transcriptional changes of selected genes in the absence of iron in response to oxygen availability in *S. flexneri*

Gene	Function	Fold-change [a] Range	Average	S.D.
Transcripts elevated aerobically				
Metabolic genes				
ndk	Aerobic ribonucleotide reductase	1.0-2.8	1.8	0.7
nrdA	Aerobic ribonucleotide reductase	1.6-2.2	2.0	0.3
nrdI	Aerobic ribonucleotide reductase	1.7-2.6	2.1	0.4
atpC	F_1F_0 ATPase	2.5-3.2	2.8[b]	
atpD	F_1F_0 ATPase	2.1-3.1	2.7	0.4
atpG	F_1F_0 ATPase	1.5-2.4	1.9	0.4
atpA	F_1F_0 ATPase	1.8-3.6	2.7	0.8
atpH	F_1F_0 ATPase	2.3-5.3	3.8	1.5
atpF	F_1F_0 ATPase	2.0-5.3	3.1	1.9
aceE	Pyruvate dehydrogenase	4.4-6.7	5.5[b]	
lpdA	Pyruvate dehydrogenase	3.0-9.1	5.7	3.1
acnA	Aconitase	2.0-7.5	4.8[b]	
mdh	Malate dehydrogenase	3.8-8.5	5.8	2.4
Stress response genes				
iscA	Fe-S cluster formation	1.0-3.6	2.5	1.3

iscU	Fe-S cluster formation	2.6-15.3	9.0[b]	
iscR	Fe-S cluster formation	1.9-20.4	8.2	10.5
sufE	Fe-S cluster formation	1.4-2.8	2.2	0.7
sufS	Fe-S cluster formation	1.1-3.2	2.2	0.9
sufD	Fe-S cluster formation	1.0-3.0	2.1	0.9
sufC	Fe-S cluster formation	1.4-3.0	2.2	0.6
sufA	Fe-S cluster formation	1.3-2.4	1.8	0.5
sodA	Superoxide dismutase	3.1-16.3	9.7[b]	
tpx	Thiol peroxidase	3.3-8.9	5.8	2.9
zwf	Glucose-6-phosphate dehydrogenase	1.2-2.8	1.9	0.7
Iron transport genes				
iucA	Aerobactin synthesis	3.3-7.0	4.8	1.6
iucB	Aerobactin synthesis	2.5-3.9	3.0	0.7
iucC	Aerobactin synthesis	2.3-4.2	3.2	1.0
iucD	Aerobactin synthesis	4.2-6.1	5.3	0.7
iutA	Aerobactin transport	3.8-9.4	6.2	2.5
sitA	Fe/Mn acquisition	2.4-5.7	3.9	1.1
sitB	Fe/Mn acquisition	3.0-7.2	4.5	1.6
sitC	Fe/Mn acquisition	2.6-6.1	4.1	1.5
sitD	Fe/Mn acquisition	2.7-4.5	3.6	0.8
Virulence plasmid genes				
shET-2	Enterotoxin	1.4-3.1	2.0	0.8

parA	Plasmid partitioning protein	1.4-2.2	1.8	0.3
virF	TTSS master regulator	1.2-2.7	1.8	0.7
virB	TTSS transcriptional activator	1.1-2.8	1.8	0.7
mxiL	TTSS apparatus	1.1-2.2	1.6	0.5
mxiM	TTSS apparatus	1.1-3.4	2.0	1.0
mxiA	TTSS apparatus	1.1-2.2	1.7	0.4
spa33	TTSS apparatus	2.1-5.5	3.2	1.4
spa9	TTSS apparatus	1.4-2.2	1.9	0.4
spa47	TTSS ATPase	1.5-2.4	2.1	0.3
spa15	TTSS chaperone	1.4-3.0	2.0	0.6
ospD3	TTSS secreted effector	1.0-3.2	1.8	0.9
spa32	TTSS secreted needle regulator	1.3-3.4	2.2	0.9
spa13	Unknown TTSS protein	1.6-4.0	2.8	1.0
spa-orf10	Unknown TTSS protein	1.9-5.7	3.2	1.5
icsA	Actin assembly protein	1.8-2.8	2.4	0.5
s0225	Similar to addiction molecule	1.2-2.3	1.7	0.5
Transcripts elevated anaerobically				
Metabolic genes				
adhE	Alcohol dehydrogenase	1.9-3.5	2.6	0.7
pflB	Pyruvate formate lyase	1.7-3.0	2.4	0.5
hypA	Hydrogenase maturation	1.4-3.3	2.5	1.0
hypB	Hydrogenase maturation	1.5-3.6	2.7	1.1

hypC	Hydrogenase maturation	1.9-3.5	2.5	0.9
molR	Molybdate metabolism regulator	1.6-2.9	2.3	0.7
narK	Nitrate reduction	2.2-30.5	12.2	12.8
narG	Nitrate reduction	2.8-32.1	15.5	14.4
narH	Nitrate reduction	1.9-15.4	8.8	6.1
narJ	Nitrate reduction	2.4-15.7	9.5[b]	6.6
narI	Nitrate reduction	1.6-9.5	4.9	4.1
Stress response genes				
hdeB	Acid stress chaperone	1.3-3.0	2.3	0.7
hdeA	Acid stress chaperone	2.0-4.1	3.1	0.8
gadA	Glutamate decarboxylase	1.5-3.8	2.6	1.0
Iron transport genes				
feoA	Ferrous iron transport	1.3-4.3	2.7	1.0
feoB	Ferrous iron transport	1.7-3.1	2.2	0.6
feoC	Ferrous iron transport	1.2-3.0	2.2	1.0
Virulence plasmid gene				
ipaC	TTSS translocation pore	1.4-2.3	1.9	0.4

[a]Fold-change and standard deviation (S.D.) were calculated as described in Materials and Methods. These data were published previously by the author and used with permission from publisher (Boulette & Payne, 2007).
[b]Standard deviations were not available if fewer than three data sets met quality controls.

S. flexneri (Tables 5 and 6 and Appendices A and B). Transcripts elevated anaerobically in *E. coli* and during anaerobic growth of *S. flexneri* in the presence or absence of iron included the *hyp* operon, *narGHJI* operon, and *hdeA* (Table 5 and 6 and Appendices A and B). Additional transcripts that were elevated anaerobically in *E. coli* or during anaerobic growth of *S. flexneri* in limited iron included the fermentation genes *adhE* and *pfl*, *molR* encoding the molybdate metabolism regulator, and *gadA* and *hdeB* acid stress genes (Table 6 and Appendix B). Also of note, several additional anaerobic reductases and other anaerobic respiratory enzymes showed larger differences in expression in *S. flexneri* in the presence of iron than in its absence (compare Tables 5 and 6 and Appendices A and B). These arrays also showed that genes involved in fermentation and synthesis of the F_1F_0 ATPase exhibited larger aerobic and anaerobic differences in expression in the absence of iron. Since respiratory enzymes often require iron for their activity, fewer complexes for anaerobic respiration are likely to significantly impact the growth of *S. flexneri* in the absence of iron. Fermentation produces less energy for the organism than anaerobic respiration, and the need for the F_1F_0 ATPase is decreased in the absence of respiration. Thus, elevated levels of fermentation and ATPase gene expression and decreased expression of anaerobic respiratory genes during anaerobiosis in the absence of iron are expected.

Since anaerobiosis induces the expression of virulence genes in other organisms, it was important to determine whether any plasmid-encoded virulence genes of *S. flexneri* were induced during oxygen restriction. Surprisingly, several virulence plasmid genes were elevated aerobically in the absence of iron. These genes encoded various proteins of the TTSS, including the VirF and VirB TTSS activators, a chaperone (Spa15), components of the apparatus (MxiL, MxiM, MxiA, Spa33, Spa9, and Spa47), a secreted

effector (OspD3), the Spa32 needle length regulator, as well as two unknown TTSS proteins (Spa-Orf10 and Spa13). The *shET-2* gene encoding an enterotoxin protein, *parA* gene encoding a plasmid partitioning protein, *icsA* gene encoding the actin assembly protein for intercellular spread, and the *s0225* gene encoding a protein with homology to addiction molecules for plasmid maintenance were also elevated aerobically. However, only the *ipaC*-encoded TTSS effector displayed a reproducible transcriptional difference in response to oxygen depletion in the absence of iron (Table 6). Microarrays suggested *ipaC* was induced 1.9-fold upon the switch to anaerobiosis. However, real time RT-PCR analysis of the *ipaC* message did not demonstrate any significant difference in expression between aerobically and anaerobically grown cultures (data not shown). Due to conflicting transcriptional data, and since *ipaC* was the sole gene within the *ipaBCDA* operon that exhibited an anaerobic elevation in expression and numerous TTSS genes typically expressed with *ipaC* showed aerobic elevation, the regulation of *ipaC* by anaerobiosis was not pursued further.

1.3. *S. FLEXNERI* IRON ACQUISITION SYSTEMS REGULATED BY ANAEROBIOSIS

Several genes that are repressed by Fur in *S. flexneri* showed greater aerobic and anaerobic differences in the absence of iron. In iron-replete conditions, acquisition of iron is repressed due to the activation of Fur by free Fe^{2+}. Therefore, in the absence of iron, it became apparent that the transcription of several iron acquisition genes responded to oxygen availability. *feo* transcription was induced more than two-fold anaerobically (Table 6 and Appendix B). Anaerobic elevation of the *feo* transcript is consistent with the role of Feo in ferrous iron acquisition and with data from *E. coli* in which Fnr was

shown to induce *feo* expression (Kammler *et al.*, 1993). Transcripts of the *sit* genes were less abundant anaerobically than aerobically, which was also observed in *S. enterica* serovar Typhimurium (Ikeda *et al.*, 2005). Similarly, the *iucABCD iutA* operon showed a decrease in expression during anaerobic conditions (Table 6 and Appendix B).

Transcriptional regulation of the iron acquisition loci by oxygen availability was verified by GFP reporter fusion assays. Transcriptional *gfp*-reporter fusions in the low-copy pLR29 vector were made using promoters upstream of the *feoABC*, *sitABCD*, and *iucABCD iutA* operons (Fig. 11). Wild type *S. flexneri* containing each reporter fusion was grown aerobically or anaerobically to compare the relative promoter activity under these conditions. The reporter data confirmed that the *iuc* and *sit* promoters were more active aerobically than anaerobically, while the *feo* promoter was more active anaerobically (Fig. 12). The specific substrates of the *S. flexneri* Sit system have not yet been determined. However, Feo transports ferrous iron, which is more abundant anaerobically, while the Iuc/Iut system mediates acquisition of ferrous iron, the predominant form of iron under aerobic conditions. Therefore, the regulation of *feo* and *iuc* by oxygen availability in *S. flexneri* suggests that iron transporters are synthesized during the conditions appropriate for their substrate availability.

2. Measuring *Shigella* Virulence in an Anaerobic Environment

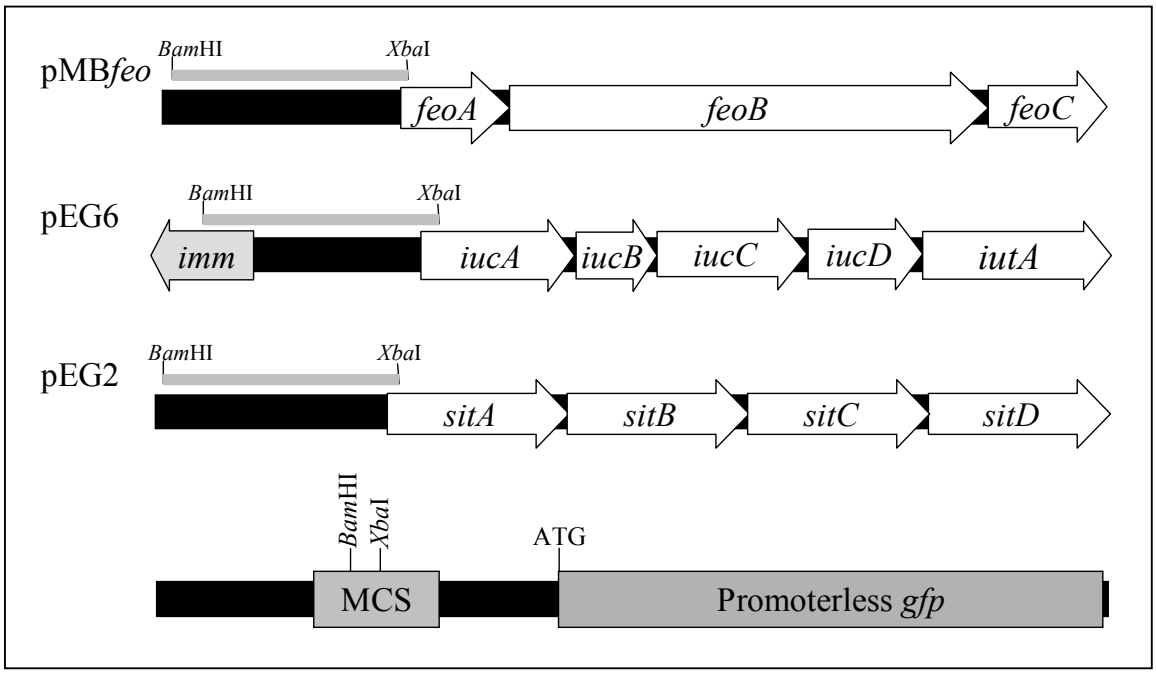


Figure 11. GFP-reporter fusions to *feo*, *iuc*, and *sit* promoters

The *feo*, *iuc/iut*, and *sit* genomic regions, as well as the multiple cloning site (MCS) and promoterless *gfp* of the pLR29 vector, are depicted. The pMB*feo* (*feo*), pEG6(*iuc/iut*), and pEG2 (*sit*) promoter regions amplified by PCR, including the 5' *Bam*HI and 3' *Xba*I restriction sites that were added for cloning into the MCS, are denoted above their corresponding genomic regions.

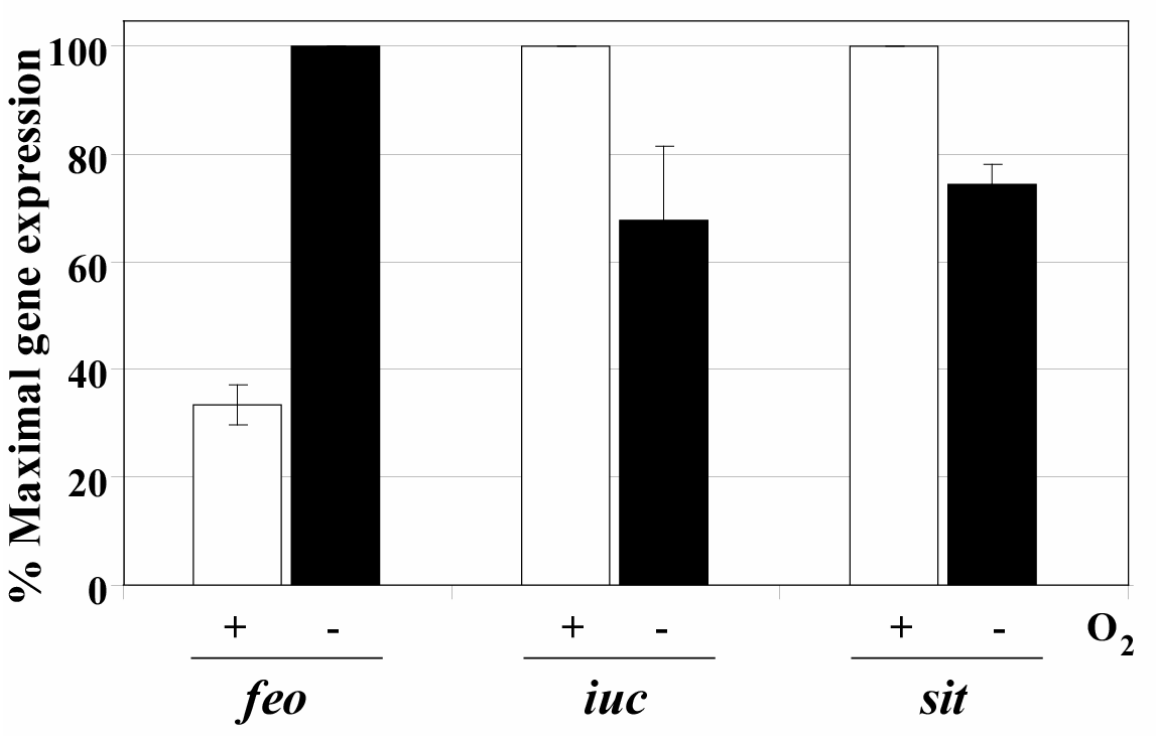


Figure 12. Effect of O_2 on expression of *gfp* fused to iron transport gene promoters

The avirulent wild type *S. flexneri* (SA101) containing promoter-*gfp* fusion plasmids pMB*feo* (*feo*), pEG6 (*iuc*), or pEG2 (*sit*) was grown in the presence or absence of oxygen, and fluorescence was measured. For each promoter, the condition with the highest relative fluorescence value was set to 100%. Error bars represent one standard deviation determined from three independent experiments. The data in this figure were published previously by the author and used with permission from publisher (Boulette & Payne, 2007).

Determining plaque formation in epithelial cell monolayers is the standard assay for measuring epithelial cell invasion, intracellular growth, and intercellular spread of *S. flexneri*, all of which are required steps for *S. flexneri* pathogenesis. There is a correlation between a plaque-null or reduced plaque phenotype and avirulence, because the bacteria exhibiting that phenotype are unable to perform the necessary steps for infection. However, the plaque assay is not a true virulence test since there are some mutants that do not cause disease in the human even though they are able to form plaques in cell monolayers. This implies that the virulence in the host requires factors which are not typically measured in the plaque assay.

2.1. DESIGNING THE ANAEROBIC PLAQUE ASSAY

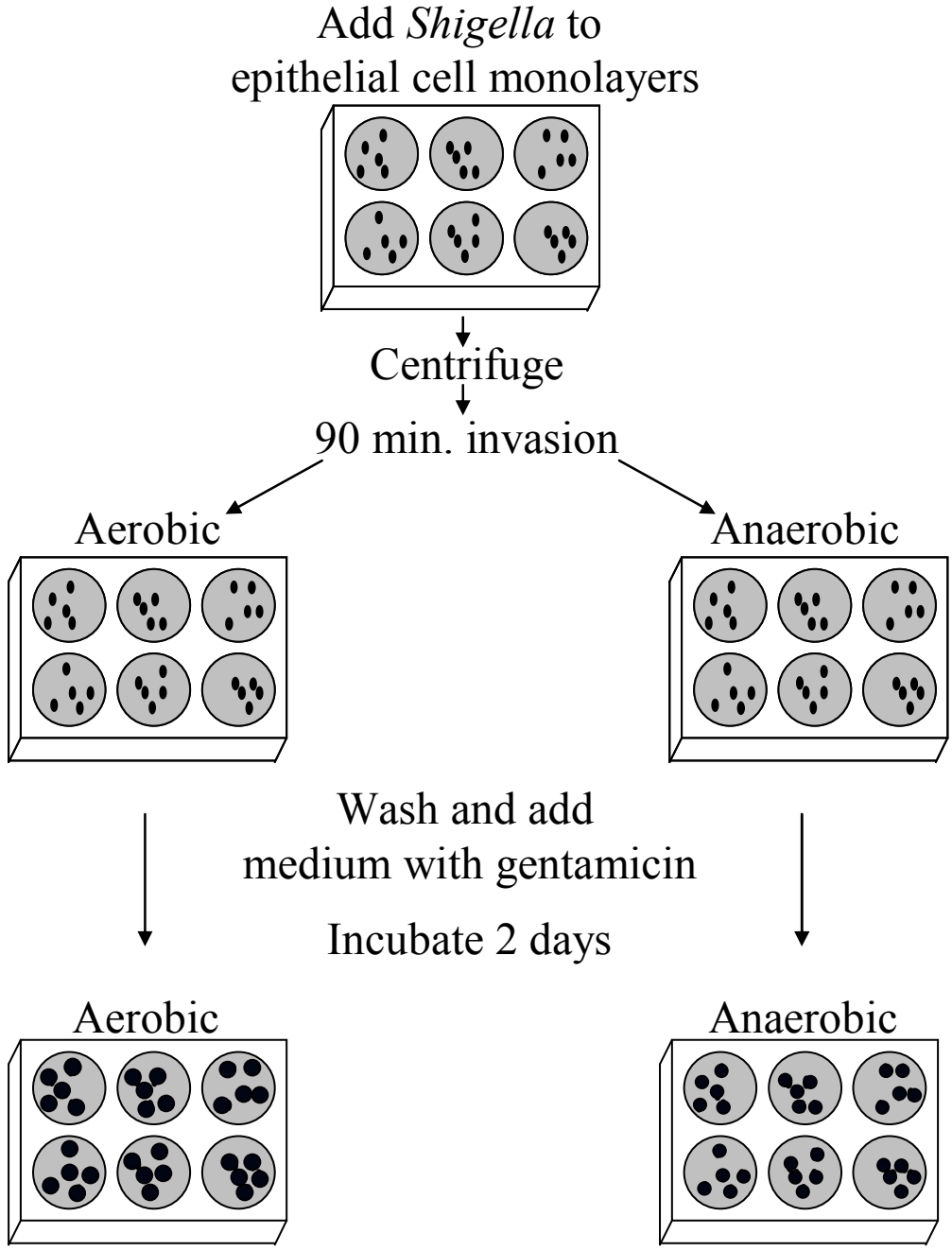
While the plaque assay (Oaks *et al.*, 1985) has been helpful in identifying genes involved in invasion, spread, intracellular multiplication, and intercellular spread, it is performed under aerobic conditions and may not accurately reflect the anaerobic environment of the human colon. Therefore, I have modified the plaque assay to assess the importance of anaerobic growth in plaque formation. Additionally, plaque assays can be performed in microaerobic pouches to determine differences in plaque formation between low-oxygen environments and those devoid of oxygen. The initial steps of the plaque assay are performed according to the standard protocol ((Materials and Methods), but the incubation conditions and medium are altered.

To compare aerobic plaque formation with plaques formed during anoxic conditions (Fig. 13), cultured human epithelial cells were grown on six-well plates to near-confluence. The epithelial cells were then infected with *Shigella* and the plates were centrifuged to allow the bacteria to localize on the epithelial surface. After centrifugation, the plates were incubated for 60-90 minutes either aerobically or in anaerobic pouches to allow bacterial invasion of the eukaryotic cells. The wells were then washed several times to remove any extracellular bacteria, and medium supplemented with HEPES buffer, glucose, and gentamicin was added. HEPES buffer was added since acidification of the medium, which leads to instability of the epithelial monolayer, occurs more rapidly during anaerobic incubation than during growth in oxidative conditions. After medium was added, the plates were incubated for two days either in the presence of oxygen or in the anaerobic pouches. During this incubation, the antibiotic gentamicin was added to ensure that only the bacteria that have entered the epithelial cells in the allotted invasion period induce lysis of the epithelial cells after successful intracellular growth and spread to adjacent cells. The medium was then aspirated and the monolayer was stained to visualize plaque numbers and sizes.

2.2. Differences in Iron Acquisition Demonstrate Significance of the Anaerobic Model of Infection

Mutants defective in one or more iron transport systems (Lawlor *et al.*, 1987; Runyen-Janecky *et al.*, 2003) were tested in aerobic and anaerobic plaque assays to determine the role of each iron transport system in plaque formation in the presence or absence of oxygen (Fig. 14 and data not shown). As shown in Figure 14, wild type *S.*

Figure 13. Flowchart demonstrating aerobic and anaerobic plaque assays in six-well plates



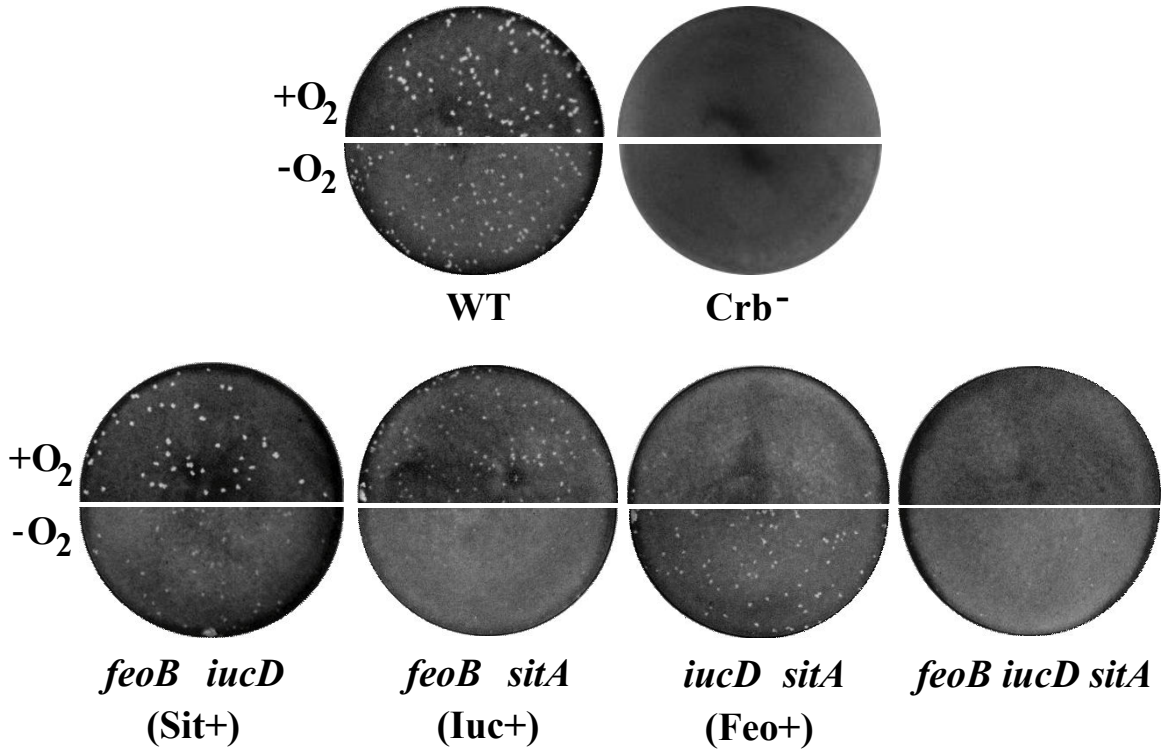


Figure 14. Plaque formation by *S. flexneri* under aerobic and anaerobic conditions

Monolayers containing Henle cells were infected with *S. flexneri* wild type (SA100), avirulent Crb$^-$ mutant (SA101), *feoB iucD* mutant (SA192), *feoB sitA* mutant (SM191), *iucD sitA* mutant (SA167), and *feoB iucD sitA* mutant (SM193) strains. The six-well plates were incubated for 2 days in medium containing gentamicin under either aerobic (upper half) or anaerobic (lower half) conditions, and wells were stained to visualize plaque formation. The data in this figure were published previously by the author and used with permission from the publisher (Boulette & Payne, 2007).

flexneri formed plaques under both aerobic and anaerobic conditions, whereas the avirulent control strain was unable to form plaques in either condition. All strains expressing at least two iron transport systems were able to form plaques in either environment (data not shown). A strain in which the Feo system (*iuc sit* double mutant) was the only functional iron transport system formed plaques anaerobically but not under aerobic conditions, while the mutant containing only a functional aerobactin system (*feo sit* double mutant) formed plaques solely under aerobic conditions. The strain with only the Sit system (*feo iuc* mutant) formed plaques in both conditions, although it formed small plaques anaerobically. The triple mutant, which does not form plaques under aerobic conditions (Runyen-Janecky *et al.*, 2003), also was unable to form plaques anaerobically. This indicates that the strain does not contain an unidentified iron transport system that functions only in the anaerobic environment. These data indicated that different iron transport systems were functioning to provide iron under different environmental conditions and suggested that the factors influencing their expression may be important for *S. flexneri* survival within the host.

To determine whether regulators of iron acquisition systems are important during plaque formation under aerobic and anaerobic conditions, the effect of Fur on the ability of *S. flexneri* to form plaques was then investigated. A *fur* mutant was constructed previously (Oglesby *et al.*, 2005), and this strain, as well as a wild type strain expressing *fur* from an IPTG-inducible plasmid, was assayed for aerobic and anaerobic plaque formation in the Henle cell model of infection. The data in Figure 15 demonstrate that over-expression of *fur* prevents plaque formation, while a mutation in *fur* reduces the number of plaques formed (Payne *et al.*, 2006). The effects of altered expression of *fur* on plaque formation occurred in both aerobic and anaerobic environments (Fig. 15),

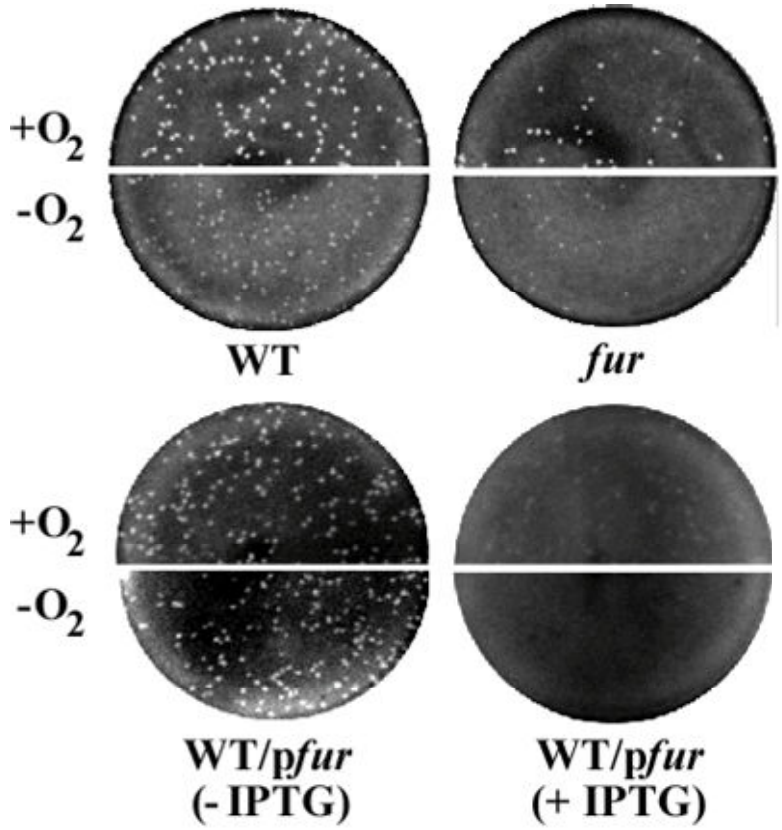


Figure 15. Fur is important for plaque formation by *S. flexneri*

Henle cell monolayers were infected with *S. flexneri* wild type (SM100) and *fur* mutant (SM1301), and SM100/p*fur* with and without 50 μM IPTG. Plates were incubated for two days in medium containing gentamicin under either aerobic (upper half) or anaerobic (lower half) conditions and differentially stained to visualize plaque formation. The data in this figure were published previously by the author and used with permission from the publisher (Boulette & Payne, 2007).

indicating that proper regulation of *fur* is important for the virulence of *S. flexneri* regardless of oxygen availability.

3. Characterization of *S. flexneri arcA* and *fnr* Mutants

The global anaerobic transcription factors ArcA and Fnr induce virulence gene expression in numerous pathogenic bacteria. To determine whether these regulators play a role in *S. flexneri* virulence, *S. flexneri arcA* and *fnr* mutant strains were made. Growth assays were performed with these strains to evaluate their growth prior to assessing their effects on transcriptional regulation and virulence.

3.1. GROWTH OF *ARCA* AND *FNR* MUTANTS

Growth of the *arcA* and *fnr* single and *arcA fnr* double mutants was assayed for initial characterization of these mutations in *Shigella*. The *fnr* mutant grew similarly to wild type when cultured aerobically in LB broth. There was a slight reduction in growth of the *arcA* and *arcA fnr* mutants (Figure 16). Since ArcA and Fnr regulate central metabolic enzymes during anaerobiosis, the wild type and *fnr*, *arcA*, and *arcA fnr* mutants were also grown anaerobically on LB agar plates with glucose as a fermentable carbon source or with glycerol as a non-fermentable carbon source with nitrate, nitrite, or

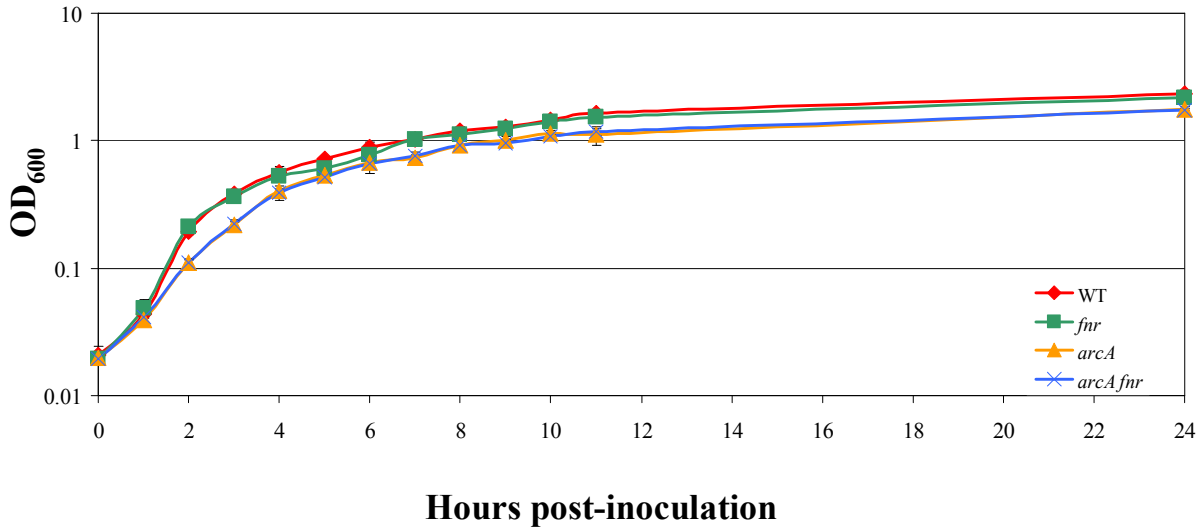


Figure 16. Aerobic growth of *S. flexneri fnr* and *arcA* mutants

The growth of *S. flexneri* wild type (SA100) and *fnr* and *arcA* single and double mutants (MBF100, MBF200, and MBF300) was monitored by reading the optical density of culture at 600nm (OD_{600}) over a 24 hour period. Bacterial cultures were inoculated from overnight cultures to the same OD_{600} and grown aerobically in LB broth. Error bars represent one standard deviation from experiments performed in triplicate.

DMSO supplemented for use as electron acceptors (Table 7). The *arcA* mutant grew similarly to wild type on medium with glycerol and nitrate or DMSO, but formed smaller colonies on glucose or glycerol with nitrite. The *fnr* and *arcA fnr* mutants formed smaller colonies than wild type on all media tested, and the double mutant was unable to grow on plates containing glycerol with nitrite. The reduced growth of *arcA* and *fnr* mutants on glucose was expected since both of these transcription factors induce genes involved in fermentation. Fnr also participates in activation of the reductases for the anaerobic electron acceptors nitrate, nitrite, and DMSO. As a result, poor growth of *fnr* on these respiratory substrates was also anticipated. However, the nitrate reductases are also induced by nitrate responsive regulators, which could explain why the *fnr* mutant still grew well on nitrate. While ArcA does not induce nitrite reduction, it does play a role in the nitrosative stress response, which may be the reason for the poor growth of the *arcA* mutant in the presence of this anaerobic electron acceptor.

3.2. EFFECTS OF *ARCA* AND *FNR* MUTATIONS ON PLAQUE FORMATION

To determine whether ArcA or Fnr is important for *S. flexneri* virulence, we tested the effect of mutations in these regulatory genes in the plaque assay under aerobic and anaerobic conditions (Fig. 17). Aerobically, plaque formation by *fnr*, *arcA*, and *arcA fnr* mutants was similar to wild type. Although the *arcA* and *fnr* single mutants formed plaques anaerobically, albeit with a slight reduction in plaque size, the *arcA fnr* double mutant did not form plaques in the absence of oxygen (Fig. 17). However, plaques were formed if the Henle cells infected with *arcA fnr* were exposed to oxygen following the

Table 7. Colony size of *S. flexneri arcA* and *fnr* mutants grown anaerobically

SA100	Growth[a] on LB Agar Supplemented with:			
Genotype	**Glucose**	**Glycerol + $NaNO_3$**	**Glycerol + $NaNO_2$**	**Glycerol + DMSO**
WT	2 (0.2)	1.8 (0.2)	1.4 (0.2)	1.7 (0.3)
fnr	1.0 (0.1)	1.6 (0.1)	0.7 (0.1)	1.3 (0.1)
arcA	1.2 (0.1)	2.0 (0.2)	0.9 (0.1)	1.8 (0.2)
arcA fnr	1.0 (0.1)	1.1 (0.1)	0.0 (0)	0.9 (0.1)

[a] Average diameter of 10 colonies in mm after 2 days growth. Standard deviation is indicated in parentheses.

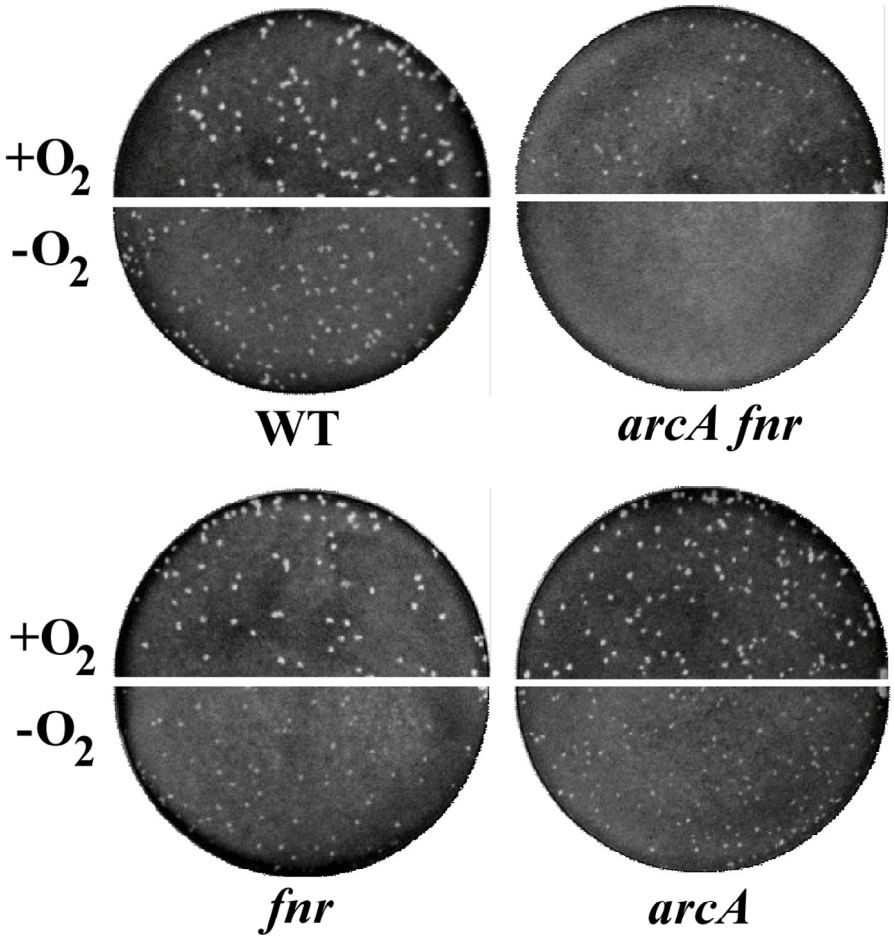


Figure 17. ArcA and Fnr are important for anaerobic plaque formation by *S. flexneri*

Henle cell monolayers were infected with *S. flexneri* wild type (SA100) and anaerobic regulatory mutants MBF100 (*fnr*), MBF200 (*arcA*), and MBF300 (*arcA fnr*). Plates were incubated for 2 days in medium containing gentamicin under either aerobic (upper half) or anaerobic (lower half) conditions and differentially stained to visualize plaque formation. The data in this figure were published previously by the author and used with permission from the publisher (Boulette & Payne, 2007).

usual period of anaerobic incubation (data not shown). This indicates that the *arcA fnr* mutant could invade and survive in the intracellular environment, but was defective in either intracellular growth or cell to cell spread. Plaque formation was also restored under anaerobic conditions by complementation with either *fnr* or *arcA* on a plasmid. These data indicate that ArcA and Fnr exhibit a partial redundancy in their ability to regulate one or more of the required steps for plaque formation.

3.3. EFFECTS OF *ARCA* AND *FNR* MUTATIONS ON *S. FLEXNERI* GENE EXPRESSION

In *E. coli* and other organisms, ArcA and Fnr are global regulators that are activated during anaerobiosis. Both of these transcription factors are capable of acting as repressors or activators, regulating expression of target genes, which participate in respiration and other aspects of carbon and energy metabolism. However, they also contribute to the regulation of virulence genes in some organisms. Since the *S. flexneri arcA fnr* mutant exhibited a virulence defect in plaque assays, the effects of the loss of these transcriptional regulators on *S. flexneri* transcription was determined. To do so, microarrays, transcriptional reporter assays, and RT-PCR were performed.

3.3.1. Regulation of Gene Expression by ArcA

Microarrays comparing the anaerobic transcriptional profiles of *S. flexneri* wild type and the *arcA* mutant were performed to identify possible ArcA targets in *Shigella*

(Table 8 and Appendix C). Genes showing increased expression in wild type *E. coli* compared to the *arcA* mutant included the *rpmF-fabG* operon encoding proteins involved in translation and fatty acid biosynthesis (Liu & De Wulf, 2004), the *rpsJ-rplQ* operon encoding transcription and translation proteins (Salmon *et al.*, 2005), and the *gadB* gene encoding glutamate decarboxylase for acid resistance (Salmon *et al.*, 2005). In *S. flexneri*, these operons also showed a similar pattern of expression (Table 8 and Appendix C), as did an *S. flexneri* transcript encoding a hypothetical protein of unknown function that appeared to be highly induced by ArcA in *E. coli* (Table 8 and Appendix C, represented on the array by two distinct oligonucleotide sequences annotated *b1172* and *s1245*) (Salmon *et al.*, 2005). In addition, the *S. flexneri feoB* transcript was elevated in wild type compared to the *arcA* mutant, which was not observed in *E. coli*. The ArcA-dependent regulation of *feoB* suggests that ArcA may induce this transcript in iron-limiting, anaerobic conditions.

Genes whose expression decreased in both *E. coli* and *S. flexneri* wild type compared to the *arcA* mutant included the acetyl-CoA synthetase (*acs*), lactate permease (*lldPD*), pyruvate dehydrogenase complex (*lpd-ace*) involved in fermentation, and several TCA cycle enzymes (*acnA*, *acnB*, *gltA*, *icdA*, *mdh*, *sdhCDAB*, *b0725*, and *sucBCD*) (Table 8 and Appendix C) (Iuchi & Lin, 1988; Liu & De Wulf, 2004; Salmon *et al.*, 2005). Also among this group were a glycolytic enzyme (*gpmA*), oligopeptide permease (*oppA*), thiol peroxidase (*tpx*), histidine binding protein (*hisJ*), and a putative σ^{54} modulator (*yfiA*) (Salmon *et al.*, 2005). Two genes involved in Fe^{3+} acquisition in *E. coli*, *fhuF*, encoding a Fe^{3+} reductase, and *fecC*, encoding a portion of the ferric citrate permease that is not functional in SA100, appeared to be increased anaerobically in the *S. flexneri arcA* mutant relative to wild type, indicating putative ArcA repression of these

Table 8. Microarrays demonstrate transcriptional changes between *S. flexneri* wild type and the *arcA* mutant when grown anaerobically in iron deplete media

Gene	Function	Fold-change [a]		
		Range	Average	S.D.
Transcripts elevated anaerobically in WT				
ArcA-responsive metabolic genes				
fabG	Fatty acid biosynthesis	2.2-2.4	2.3	0.1
rpoA	RNA polymerase core	2.2-3.6	2.9[b]	
rpmF	Ribosomal protein	1.7-4.1	3.2	1.3
rpsJ	Ribosomal protein	1.2-6.7	3.5	2.8
rplD	Ribosomal protein	1.0-6.9	4.6	3.2
rplW	Ribosomal protein	2.9-3.4	3.1[b]	
rplB	Ribosomal protein	1.4-6.0	3.9	2.4
rpsS	Ribosomal protein	1.4-5.4	3.3	2.0
rplV	Ribosomal protein	1.9-6.3	4.3	2.3
rplP	Ribosomal protein	1.1-4.5	3.1	1.7
rpsQ	Ribosomal protein	2.7-3.9	3.3[b]	
rplN	Ribosomal protein	2.1-3.1	2.6[b]	
rplE	Ribosomal protein	1.3-4.7	2.8	1.7
rpsH	Ribosomal protein	1.3-5.6	3.8	2.2
rplF	Ribosomal protein	1.5-5.6	3.1	2.2

rplR	Ribosomal protein	1.7-6.9	4.7	2.7
rpmD	Ribosomal protein	1.2-5.0	3.3	1.9
rplO	Ribosomal protein	1.8-6.4	4.1	2.3
prlA	Ribosomal and secretion protein	1.6-4.8	3.4	1.7
rpmJ	Ribosomal protein	2.3-4.6	3.5[b]	
rpsM	Ribosomal protein	1.3-5.1	3.4	1.9
rpsK	Ribosomal protein	1.5-5.4	3.4	1.9
rpsD	Ribosomal protein	1.9-5.5	4.1	1.9
rplQ	Ribosomal protein	1.2-3.2	2.5	1.1
ArcA-responsive stress response gene				
gadB	Glutamate decarboxylase	2.0-8.3	5.2[b]	
ArcA-responsive iron transport gene				
feoB	Ferrous iron acquisition	2.0-2.9	2.5[b]	
ArcA-responsive unknown genes				
b1172	Hypothetical protein	1.4-3.6	2.4	1.2
s1245	Homology to *b1172*	2.2-6.2	3.5	2.3
Transcripts elevated anaerobically in *arcA*				
ArcA-responsive metabolic genes				
acs	Acetyl Co-A synthetase	6.1-11.6	8.8	2.8
gpmA	Phosphoglyceromutase	1.6-2.9	2.2	0.6
hisJ	Histidine binding protein	2.3-2.4	2.4[b]	
lldP	Lactate permease	6.9-20.0	13.2	6.6

lldD	Lactate permease	7.9-13.7	10.3	3.0
oppA	Oligopeptide permease	1.8-3.3	2.5	0.8
aceF	Pyruvate dehydrogenase	4.1-5.8	4.7	0.9
lpdA	Pyruvate dehydrogenase	8.8-9.7	9.2	0.4
yfiA	Sigma 54 modulator	1.3-2.9	2.3	0.8
acnA	Aconitase	8.1-10.2	9.1[b]	
acnB	Aconitase	2.7-4.2	3.4[b]	
icdA	Isocitrate dehydrogenase	6.6-29.3	18.0[b]	
gltA	Citrate synthase	14.5-19.5	16.4	2.7
mdh	Malate dehydrogenase	3.2-5.7	4.5[b]	
sdhC	Succinate dehydrogenase	11.4-18.3	14.8[b]	
sdhD	Succinate dehydrogenase	11.6-80.1	40.0	35.7
sdhA	Succinate dehydrogenase	12.3-38.3	26.2	13.1
sdhB	Succinate dehydrogenase	8.3-24.0	15.7	7.9
b0725	Transcribed with *sdh*/*suc*	19.4-22.8	21.1[b]	
sucB	Succinyl transferase	9.9-15.3	13.5	3.1
sucC	Succinyl transferase	9.4-16.6	13.0	3.6
sucD	Succinyl transferase	11.4-18.5	15.0[b]	
ArcA-responsive stress response genes				
katG	Catalase	6.9-8.2	7.6	0.7
tpx	Thiol peroxidase	3.1-6.6	4.9	1.8
ArcA-responsive iron transport genes				

fecC	Ferric citrate transport (inactive)	2.2-2.3	2.3[b]	
fhuF	Ferric reductase	1.2-3.8	2.9	1.5
ArcA-responsive virulence plasmid genes				
mxiK	TTSS apparatus	2.8-3.7	3.2[b]	

[a]Fold-change and standard deviation (S.D.) were calculated as described in Materials and Methods.
[b]Standard deviations were not available if fewer than three data sets met quality controls.

loci. Additionally, *mxiK*, involved in the assembly of the TTSS apparatus, was elevated in the *arcA* strain. As *mxiK* was the sole gene of its large operon exhibiting consistent regulation in these arrays, it is unlikely that ArcA has a direct effect on its transcription.

3.3.2. Regulation of Gene Expression by Fnr

Although the *S. flexneri fnr* mutant did not exhibit a defect in plaque formation under anaerobic conditions, the *arcA fnr* double mutant was completely defective in plaque formation in the absence of oxygen. Therefore, the lack of Fnr contributed to the inability of *arcA fnr* to form plaques, suggesting that Fnr must be involved in regulation of genes that affect *S. flexneri* virulence. Microarrays were performed that compared the transition of wild type and the *fnr* mutant to anaerobiosis (Table 9 and Appendix D). Since active Fnr requires an iron-sulfur cluster, these assays were performed in the presence of added iron.

Overall, fewer genes passed the quality standards in the microarrays, so the transcriptional profiles of these strains are incomplete. Additionally, growth in the presence of iron would prevent the identification of expression differences in wild type and *fnr* of genes repressed by Fur, such as the *feo* genes for ferrous iron acquisition, which are known to also be activated by Fnr. However, several similarities were noted between *E. coli* and *S. flexneri* arrays of anaerobically grown wild type and *fnr*. Transcripts more abundant in wild type *S. flexneri* than in the *fnr* mutant that displayed a similar pattern of regulation in *E. coli* were those of the *narGHJI* operon

Table 9. Microarrays demonstrate transcriptional changes between *S. flexneri* wild type and the *fnr* mutant when grown anaerobically in iron-rich media

Gene	Function	Fold-change [a] Range	Average	S.D.
Transcripts elevated anaerobically in WT				
Fnr-responsive metabolic genes				
narG	Nitrate reduction	2.1-4.9	3.5[b]	
narH	Nitrate reduction	1.5-3.8	2.6	1.2
narI	Nitrate reduction	2.3-7.0	4.1	2.5
atpD	F_1F_0 ATPase	2.3-3.1	2.7[b]	
Fnr-responsive virulence plasmid gene				
mxiD	TTSS apparatus	3.0-3.4	3.2[b]	
Transcripts elevated anaerobically in *fnr*				
Fnr-responsive metabolic genes				
cydB	Cytochrome D oxidase	2.4-2.7	2.6[b]	
ndh	NADH dehydrogenase	1.8-3.7	2.9	1.0
manY	Mannose PTS system	2.3-3.2	2.7[b]	
Fnr-responsive virulence plasmid genes				
s0016	Hypothetical receptor kinase	2.5-2.8	2.7[b]	
ospC3	TTSS secreted effector	2.5-3.7	3.1[b]	

[a]Fold-change and standard deviation (S.D.) were calculated as described in Materials and Methods.
[b]Standard deviations were not available if fewer than three data sets met quality controls.

(Walker & DeMoss, 1991) and the F_1F_0 ATPase (Kasimoglu *et al.*, 1996). The *E. coli* microarrays had previously shown that genes encoding the aerobic respiratory enzymes cytochrome D terminal oxidase (*cydB*) and NADH dehydrogenase (*ndh*), as well as the mannose PTS system (*manY*), were more abundant under anaerobic conditions in the *fnr* mutant relative to wild type (Kang *et al.*, 2005; Salmon *et al.*, 2003).

The expression of some virulence plasmid genes appeared to be influenced by Fnr. The *mxiD* gene, encoding a portion of the TTSS apparatus, was elevated in wild type compared to the *fnr* mutant. This regulation is likely insignificant given *mxiD* is part of a larger operon for which additional transcriptional changes were not observed. Two virulence plasmid genes displayed reduced expression in wild type *S. flexneri* relative to the *fnr* mutant. These virulence genes included *ospC3*, encoding a TTSS effector, and *s0016*, encoding a hypothetical receptor kinase. Since it is probable that genes necessary for virulence would be elevated in a virulent strain compared to an avirulent one, it is unlikely that an Fnr-dependent repression of either of these virulence plasmid genes would account for the anaerobic difference in virulence between *S. flexneri* wild type and the *arcA fnr* mutant.

3.3.3. Regulation of Gene Expression by ArcA and Fnr

Since the *arcA fnr* double mutant was defective in anaerobic plaque formation, it was likely that one or more genes required for virulence were regulated by ArcA and Fnr. To identify possible virulence gene targets of these transcription factors and to observe

the overall anaerobic transcriptional differences between *S. flexneri* wild type and the *arcA fnr* mutant, microarray analysis comparing these strains was performed. The wild type and *arcA fnr* mutant were isolated after the transition to anaerobiosis in both iron-replete and iron-deplete media to determine the transcriptional profiles of these strains.

The arrays comparing gene expression between anaerobically grown *S. flexneri* wild type and *arcA fnr* strains in the presence of abundant iron showed a large number of genes that were significantly and consistently regulated (Table 10 and Appendix E). Numerous genes showed transcriptional changes between wild type and the *arcA fnr* mutant that were also reported in arrays comparing *E. coli* wild type with either the *fnr* or *arcA* mutant. These included genes elevated in wild type *S. flexneri* relative to the *arcA fnr* mutant that play roles in anaerobic respiration (*frdC*, *hypD*, *narL-J*), fatty acid biosynthesis (*fabG*), and transcription and translation (*rpmF*, *rplC-rpoA*). Genes with decreased expression in wild type *E. coli* relative to *arcA* or *fnr* mutants and in *S. flexneri* wild type relative to the *arcA fnr* mutant were involved in pyruvate metabolism (*lpdA-aceE*), lactate transport (*lldPRD*), the TCA cycle (*acnA*, *gltA-sucD*, *icdA*, and *mdh*), and oxidative stress (*tpx*). The F_1F_0 ATPase transcripts (*atpD-F*) were also more abundant in wild type, which may be caused by and contribute to the overall increased growth rate of this strain. Other notable genes that were elevated in wild type that contribute to energy production during anaerobic growth were *tatAB*, encoding components of the twin-arginine translocation system that localizes various anaerobic respiratory enzymes in the inner membrane and periplasm.

Table 10. Microarrays demonstrate transcriptional changes between *S. flexneri* wild type and the *arcA fnr* mutant when grown anaerobically in iron-replete media

		Fold-change [a]		
Gene	**Function**	**Range**	**Average**	**S.D.**
Transcripts elevated anaerobically in WT				
ArcA- and/or Fnr-responsive metabolic genes				
frdC	Fumarate reductase	2.5-2.9	2.7[b]	
hypD	Hydrogenase maturation	2.3-2.3	2.3[b]	
narL	Nitrate reductase regulator	2.3-3.6	3.0[b]	
narG	Nitrate reductase	6.3-20.1	13.2[b]	
narH	Nitrate reductase	2.6-22.3	15.6	11.3
narJ	Nitrate reductase	7.8-20.2	14.9	6.3
fabG	Fatty acid biosynthesis	5.8-14.5	10.9	4.5
atpD	F_1F_0 ATPase	1.9-5.7	4.3	2.1
atpG	F_1F_0 ATPase	3.8-6.9	5.4[b]	
atpA	F_1F_0 ATPase	7.7-11.2	9.4[b]	
atpH	F_1F_0 ATPase	9.5-13.2	11.4[b]	
atpF	F_1F_0 ATPase	6.5-11.3	8.9[b]	
himA	Nucleoid associated protein IHF	2.4-4.8	3.6[b]	
ompC	Outer membrane protein	3.8-14.5	10.0	5.6

hlpA	Periplasmic chaperone Skp	3.1-5.9	4.5[b]	
rpmF	Ribosomal protein	10.6-13.7	12.2	
rplC	Ribosomal protein	7.5-35.1	20.5	13.9
rplW	Ribosomal protein	26.2-44.8	35.5[b]	
rplB	Ribosomal protein	25.3-30.9	28.1[b]	
rpsS	Ribosomal protein	2.7-26.7	10.9[b]	
rplV	Ribosomal protein	6.8-31.8	19.3[b]	
rplP	Ribosomal protein	6.3-8.6	7.4[b]	
rplN	Ribosomal protein	14.4-17.1	15.7[b]	
rplE	Ribosomal protein	9.2-10.2	9.8[b]	
rpsN	Ribosomal protein	21.5-28.5	25.0[b]	
rplF	Ribosomal protein	13.8-21.3	17.5[b]	
rplR	Ribosomal protein	11.8-32.6	20.7	10.7
rplO	Ribosomal protein	28.3-28.6	28.5[b]	
prlA	Ribosomal and secretion protein	7.6-28.2	15.7	11.0
rpmJ	Ribosomal protein	15.8-22.5	19.6	3.5
rpsK	Ribosomal protein	19.4-32.9	26.1	6.8
rpsD	Ribosomal protein	9.0-31.6	20.3[b]	
rpoA	RNA polymerase core	13.9-40.3	23.0	15.0
miaA	tRNA modification enzyme	2.1-2.9	2.5[b]	
tatA	Twin-arginine translocation	4.1-6.5	5.0	1.4
tatB	Twin-arginine translocation	2.2-5.1	4.1	1.7

ArcA- and/or Fnr-responsive virulence plasmid genes

mxiD	TTSS apparatus	2.5-3.6	3.1[b]
mxiL	TTSS apparatus	3.6-4.1	3.9[b]
spa33	TTSS apparatus	3.1-8.6	5.9[b]
spa15	TTSS chaperone	3.2-7.0	5.1[b]
virB	TTSS transcriptional activator	6.2-8.6	7.4[b]
icsB	TTSS secreted effector	2.4-4.7	3.5[b]
ipgD	TTSS secreted effector	2.4-5.7	4.0[b]
ospD3	TTSS secreted effector	2.5-7.3	4.9[b]
spa32	TTSS secreted needle regulator	3.8-8.7	6.2[b]
spa-orf10	Unknown TTSS protein	2.9-6.5	4.7[b]
s0179	Unknown TTSS protein	2.0-3.1	2.5[b]
mkaD	Virulence plasmid associated gene	2.5-2.8	2.7[b]

Transcripts elevated anaerobically in *arcA fnr*

ArcA- and/or Fnr-responsive metabolic genes

lldP	Lactate permease	12.4-26.8	21.7	8.1
lldR	Lactate permease	4.0-18.6	10.1	7.6
lldD	Lactate permease	9.7-14.8	12.9	2.8
aceE	Pyruvate dehydrogenase complex	1.8-3.9	2.8	1.1
lpdA	Pyruvate dehydrogenase complex	2.8-3.4	3.0	0.4
acnA	Aconitase	1.6-9.3	4.6	4.1
gltA	Citrate synthase	5.6-16.4	9.6	5.9

icdA	Isocitrate dehydrogenase	2.3-6.0	3.9	1.9
mdh	Malate dehydrogenase	1.9-4.5	2.8	1.5
sdhC	Succinate dehydrogenase	4.8-22.9	11.2	10.1
sdhD	Succinate dehydrogenase	4.6-17.2	12.8	7.1
sdhA	Succinate dehydrogenase	8.8-17.8	11.8	5.2
sdhB	Succinate dehydrogenase	1.9-5.5	3.6	1.8
b0725	Transcribed with *gltA-sucD*	2.5-11.3	6.3	4.5
sucA	Succinyl transferase	4.8-6.5	5.5	0.9
sucB	Succinyl transferase	3.9-7.5	6.0	1.9
sucC	Succinyl transferase	2.3-7.2	5.1	2.5
sucD	Succinyl transferase	4.6-9.5	6.4	2.7
ArcA- and/or Fnr-responsive stress response gene				
tpx	Thiol peroxidase	1.8-4.5	3.0	1.4
ArcA- and/or Fnr-responsive putative virulence associated genes				
z0024	Putative fimbrial gene	2.0-2.6	2.3	0.3
z0872	Putative fimbrial gene	2.6-2.8	2.7[b]	
z3598	Putative fimbrial gene	2.6-3.3	3.0[b]	
z5109	Similar to *escD* encoding TTSS apparatus protein	2.1-2.5	2.3[b]	
z5135	Similar to *escR* encoding TTSS apparatus protein	1.8-2.4	2.1	0.3

[a]Fold-change and standard deviation (S.D.) were calculated as described in Materials and Methods.
[b]Standard deviations were not available if fewer than three data sets met quality controls.

During anaerobic growth in iron limiting medium, the wild type *S. flexneri* expressed increased levels of transcripts for anaerobic respiration (*narGJ*, *nirB*, *hypA*), fermentation (*pflB*), ferrous iron transport (*feoAB*), and the response to acid shock (*hdeAB*, *gadA*) compared to the *arcA fnr* mutant (Table 11 and Appendix F). These genes are induced by one or both of ArcA or Fnr in *E. coli*. Arrays also showed that genes repressed by ArcA and/or Fnr were more abundant in the *S. flexneri arcA fnr* mutant relative to wild type. These genes included lactate permease (*lldP*), pyruvate dehydrogenase (*lpdA*), and the TCA cycle enzymes (*gltA*, *icdA*, *mdh*, *b0725*, and *sucABCD*) (Table 11 and Appendix F). The *feoAB* iron transport genes are elevated nearly four-fold in wild type *S. flexneri* (Table 11 and Appendix F). However, the *iucD* and *iutA* genes were elevated in the *arcA fnr* mutant relative to wild type. Given the importance of the Feo system in the anaerobic plaque assay, and the defective anaerobic plaque formation by the strain with only the aerobactin transporter, the dysregulation of iron transport in the *arcA fnr* mutant may contribute to its lack of virulence under anaerobic conditions.

The transcription of several genes involved in virulence was also different between wild type and the *arcA fnr* mutant grown in the presence of iron (Table 10). Genes elevated in the wild type compared to the double mutant include *hlpA* (*skp*) for proper localization of IcsA (Purdy *et al.*, 2007), *icsB* TTSS effector involved in binding IcsA to escape autophagy (Ogawa *et al.*, 2005), *miaA* tRNA modification enzyme for VirF synthesis (Durand *et al.*, 1997), *ompC* outer membrane protein essential to virulence (Bernardini *et al.*, 1993), and *himA* (*ihf*) nucleoid associated factor involved in activation of *virF*, *virB*, and *icsA* (Porter & Dorman, 1997). Several TTSS genes of the *mxi*/*spa* locus, as well as *osp* effectors and the virulence gene activator *virB*, were also elevated in

Table 11. Microarrays demonstrate transcriptional changes between *S. flexneri* wild type and the *arcA fnr* mutant when grown anaerobically in iron-deplete media

Gene	Function	Fold-change [a]		
		Range	**Average**	**S.D.**
Transcripts elevated anaerobically in WT				
ArcA- and/or Fnr-responsive metabolic genes				
hypA	Hydrogenase maturation	1.3-6.1	3.6	2.4
narG	Nitrate reductase	1.5-11.9	6.8	5.7
narJ	Nitrate reductase	1.8-5.7	3.7	1.9
nirB	Nitrite reductase	7.5-9.4	8.5[b]	
pflB	Pyruvate formate lyase	2.2-9.9	4.6	3.6
ArcA- and/or Fnr-responsive stress response genes				
hdeA	Acid stress chaperone	6.9-38.9	17.7	18.4
gadA	Glutamate decarboxylase	4.8-5.5	5.2[b]	
ArcA- and/or Fnr-responsive iron transport genes				
feoA	Ferrous iron transport	2.0-5.8	4.2	1.6
feoB	Ferrous iron transport	2.8-4.3	3.4	0.8
ArcA- and/or Fnr-responsive virulence plasmid genes				
msbB	Acylation of LPS lipid A	1.1-2.8	1.8	0.8
s0016	Hypothetical receptor kinase	1.1-9.4	3.0	3.1
ospB	TTSS secreted effector	2.3-3.7	3.0[b]	

ospC4	TTSS secreted effector	1.2-16.7	5.2	6.5
ospD3	TTSS secreted effector	1.4-6.2	2.7	2.0
ushA	UDP-sugar hydrolase	1.1-6.2	2.7	2.4
Transcripts elevated anaerobically in *arcA fnr*				
ArcA- and/or Fnr-responsive metabolic genes				
lldP	Lactate permease	8.7-98.2	43.0	36.5
lpdA	Pyruvate dehydrogenase	1.7-14.2	7.7	6.4
gltA	Citrate synthase	4.1-31.4	20.3	14.4
icdA	Isocitrate dehydrogenase	1.8-25.5	9.8	13.6
mdh	Malate dehydrogenase	2.3-13.4	5.7	4.6
b0725	Hypothetical in *gltA-sucD* operon	9.2-18.6	12.3	5.4
sucA	Succinyl transferase	2.4-19.0	10.7[b]	
sucB	Succinyl transferase	3.0-12.0	5.4	4.4
sucC	Succinyl transferase	2.4-20.6	11.5[b]	
sucD	Succinyl transferase	6.8-19.9	13.4[b]	
ArcA- and/or Fnr-responsive iron transport genes				
iucD	Aerobactin synthesis	1.5-7.5	3.9	2.4
iutA	Aerobactin transport	1.1-7.6	3.2	2.4

[a]Fold-change and standard deviation (S.D.) were calculated as described in Materials and Methods.
[b]Standard deviations were not available if fewer than three data sets met quality controls.

the wild type relative to their expression in the *arcA fnr* strain in both the presence (Table 10) and absence (Table 11) of iron. Relatively few transcripts potentially involved in *Shigella* virulence exhibited greater expression in the *arcA fnr* double mutant compared with wild type (Table 10). These transcripts are predicted to encode fimbrial proteins, which are involved in bacterial adherence, and proteins with homology to the enteropathogenic *E. coli* TTSS basal body components EscD and EscR (Ogino *et al.*, 2006).

3.3.4. Regulation of Iron Acquisition by ArcA and Fnr

Because there appears to be anaerobic regulation of iron acquisition genes, contributions of ArcA and Fnr to this regulation were explored in detail. Transcriptional *gfp*-reporter fusions to the *iuc*, *feo*, and *sit* promoters were constructed and their activity determined in the *S. flexneri* wild type and in *arcA*, *fnr*, and *arcA fnr* mutant backgrounds during aerobic and anaerobic growth. Since Fur also regulates all of these promoters to different degrees, promoter activity was assayed under iron restriction where Fur would not be active. The *narG-gfp* fusion, which is activated by Fnr, was used as a positive control for Fnr (Fig. 18A), while the *lld-gfp* fusion, which is repressed by ArcA, was used as an ArcA control (Fig. 18B).

The activity of the *iuc* promoter fusion was the same in wild type and mutant strains when the cells were grown aerobically. The activity was slightly elevated during anaerobiosis in the *arcA* mutant (Fig. 19). These data were indicative of potential ArcA

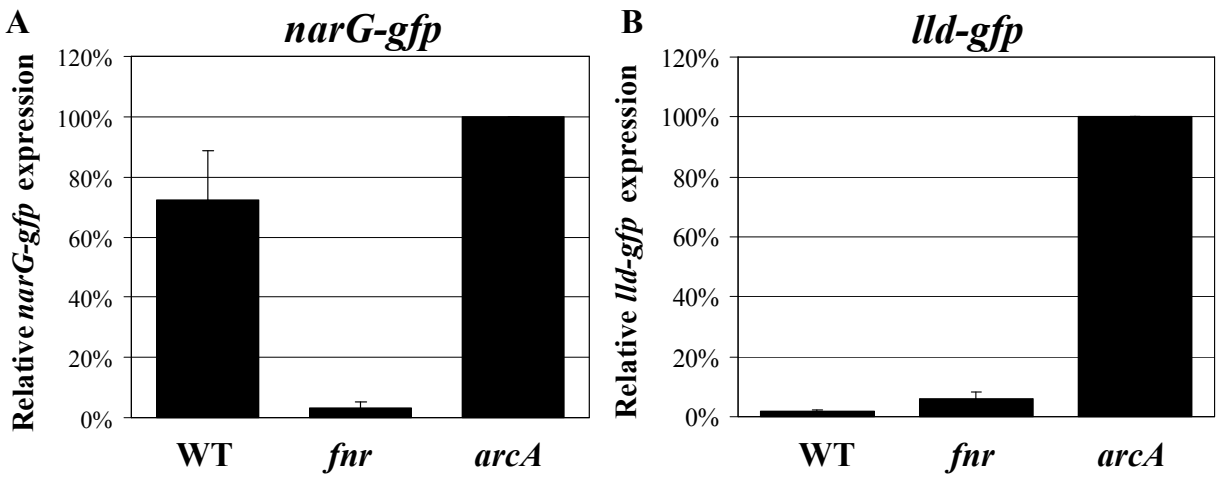


Figure 18. Expression of *narG* and *lld* promoters under anaerobic conditions

The relative *gfp* expression obtained from the **A)** *narG* (pMB*narG*) and **B)** *lld* (pMB*lld*) promoters in anaerobically grown avirulent WT (SA101), *fnr* (MBF100W), and *arcA* (MBF200W) strains are shown, with the highest relative fluorescence value set to 100%. Three independent experiments were averaged, and error bars represent one standard deviation.

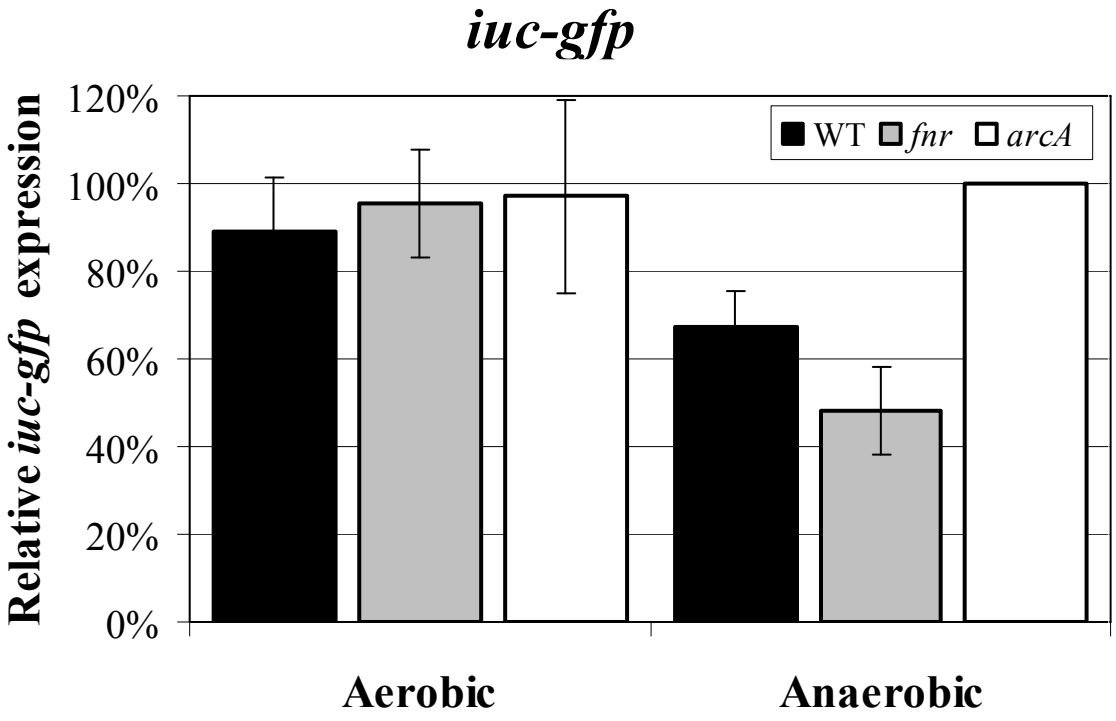


Figure 19. Expression of *iuc* promoter

The relative *gfp* expression obtained from the *iuc* promoter of pEG6 in aerobically and anaerobically grown avirulent WT (SA101), *fnr* (MBF100W), and *arcA* (MBF200W) strains are shown, with the highest relative fluorescence value set to 100%. The averages from three independent experiments are shown. Error bars represent one standard deviation. The data in this figure were published previously by the author and used with permission from the publisher (Boulette & Payne, 2007).

repression of this promoter. In contrast, the activity of the *feo* promoter was approximately half of the wild type level in the *arcA* and *fnr* single mutants and one-fifth of the wild type level in the *arcA fnr* double mutant (Fig. 20), suggesting that the anaerobic induction of *feo* transcription in the wild type strain is due to Fnr and ArcA, and that their effects are additive. The overall activity of the *sit* promoter was reduced in all strains relative to wild type (Fig. 21). However, the activity of the *sit* promoter fusion in wild type and the *fnr* mutant was greater aerobically relative to its anaerobic activity. In these strains, the ratio of aerobic to anaerobic *sit* promoter activity was 1.4 and 1.6, respectively. However, the ratio of aerobic to anaerobic activity of the *sit* promoter in the *arcA* mutant was 0.95, indicating that the elevation of expression seen under aerobic conditions in the wild type (Table 5 and Fig.12) required ArcA.

Although these three iron acquisition systems appear to comprise the repertoire of transporters contributing to aerobic and anaerobic iron uptake during plaque formation, additional systems for iron acquisition are present in this and other *Shigella* species. The regulation of two other iron transport systems, the FhuACDB hydroxamate transport system of *S. flexneri* and the Shu heme transport and utilization system of *S. dysenteriae*, was assayed by experiments using GFP reporter fusions (Fig. 22A and 23A). The anaerobic expression of *fhu* appears to be dependent on ArcA and Fnr (Fig. 22A), and potential ArcA binding sites were identified in the *fhu* promoter region (Fig. 22B). One of these putative ArcA boxes was disrupted by deletion of the 5' region between *fhuA* and *fhuAΔ* and several base pairs of another were altered in *fhuAΔalt* (Fig. 22B). Although there appears to be an ArcA-dependent loss of promoter activity by the 5' deletion, the base alterations in *fhuAΔalt* appear to have little effect (Fig. 22A). In contrast to the *fhuA* results, ArcA does not appear to play a significant role in regulation

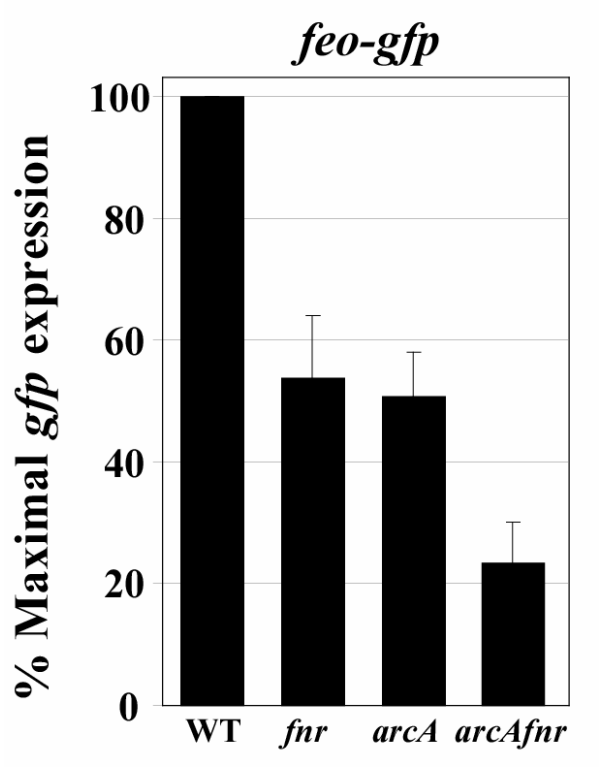


Figure 20. Expression of *feo* promoter under anaerobic conditions

The relative *gfp* expression obtained for the *feo* promoter fusion of pMB*feo* in avirulent WT (SA101), *fnr* (MBF100W), *arcA* (MBF200W), and *arcA fnr* (MBF300W) strains are shown, with the highest relative fluorescence value set to 100%. The averages from three independent experiments are shown. Error bars represent one standard deviation. The data in this figure were published previously by the author and used with permission from the publisher (Boulette & Payne, 2007).

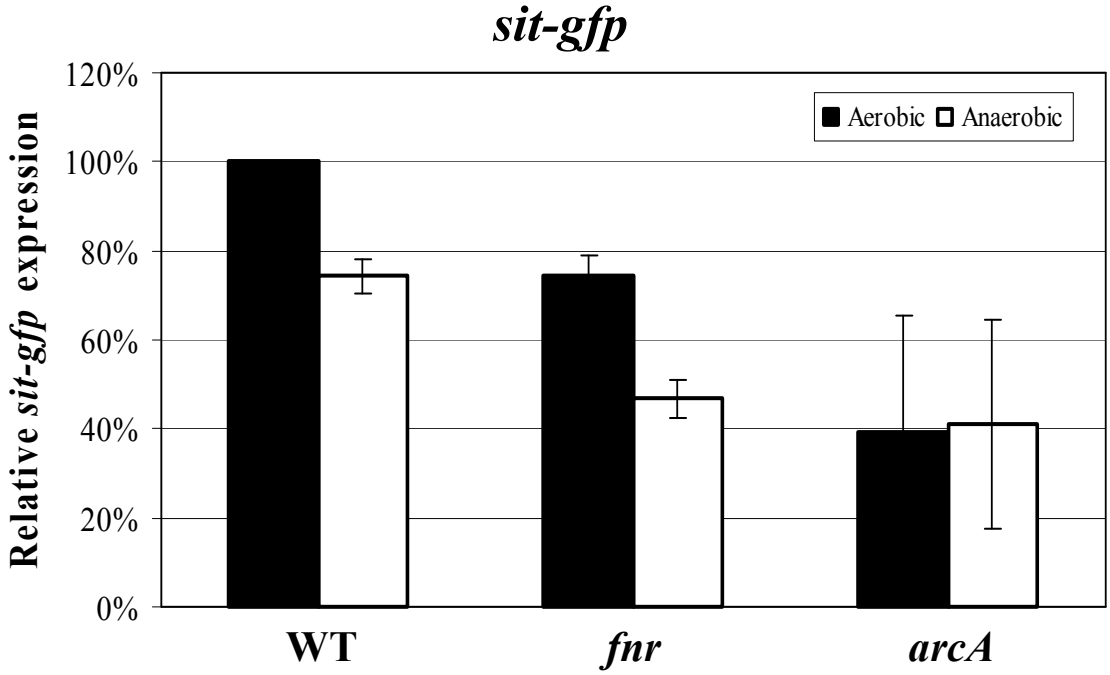
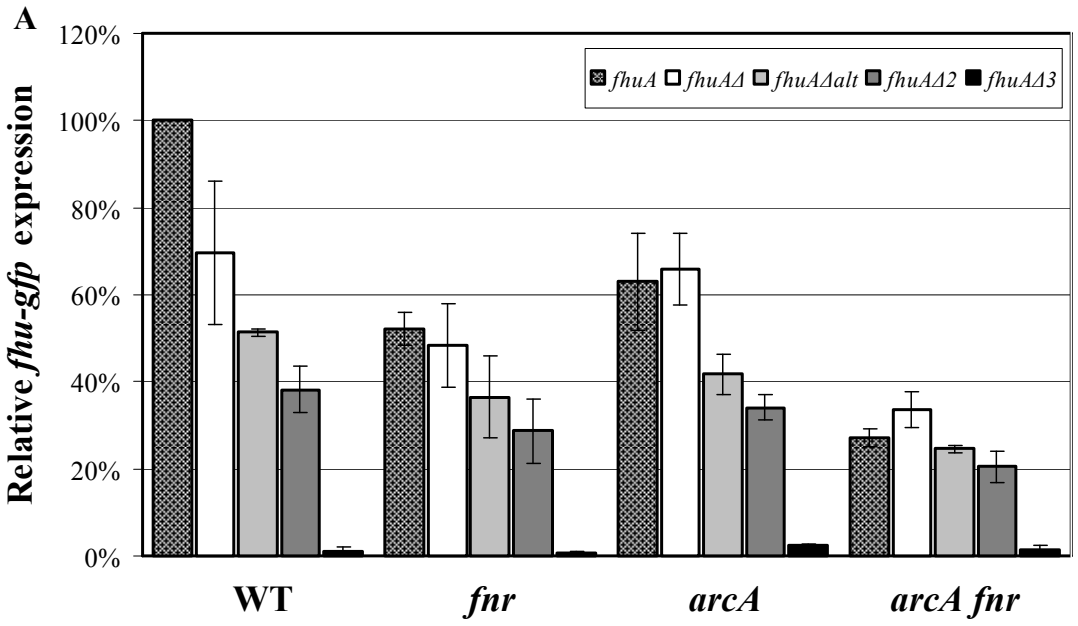


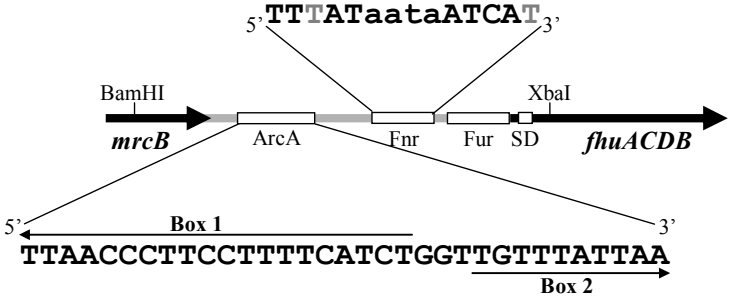
Figure 21. Aerobic and anaerobic expression of the *sit* promoter

The relative *gfp* expression obtained for the *sit* promoter fusion of pEG2 in the avirulent WT (SA101), *fnr* (MBF100), and *arcA* (MBF200) strains grown under aerobic and anaerobic conditions are shown, with the highest relative fluorescence value set to 100%. The averages from three independent experiments are shown. Error bars represent one standard deviation.

Figure 22. Anaerobic expression and organization of *fhu* promoters



B



Promoter	ArcA Box 1	ArcA Box 2	Fnr Box
fhuA	AGATGAAAAgaAAGGGTTAA	TGTTTATTAA	Yes
fhuAΔ	—	TGTTTATTAA	Yes
fhuAΔalt	—	TGCCTATCCA	Yes
fhuAΔ2	—	—	Yes
fhuAΔ3	—	—	No

Figure 22. Anaerobic expression and organization of *fhu* promoters.

A. The relative *gfp* expression obtained for *fhuA* (pEG5), *fhuAΔ* (pMB*fhuAΔ*), *fhuAΔalt* (pMB*fhuAΔalt*), *fhuΔ2* (pHG*fhuΔ2*), and *fhuΔ3* (pMB*fhuΔ3*) promoter fusions in the avirulent WT (SA101), *fnr* (MBF100), *arcA* (MBF200), and *arcA fnr* (MBF300) strains are shown, with the highest relative fluorescence value set to 100%. The averages from three independent experiments are shown. Error bars represent one standard deviation.

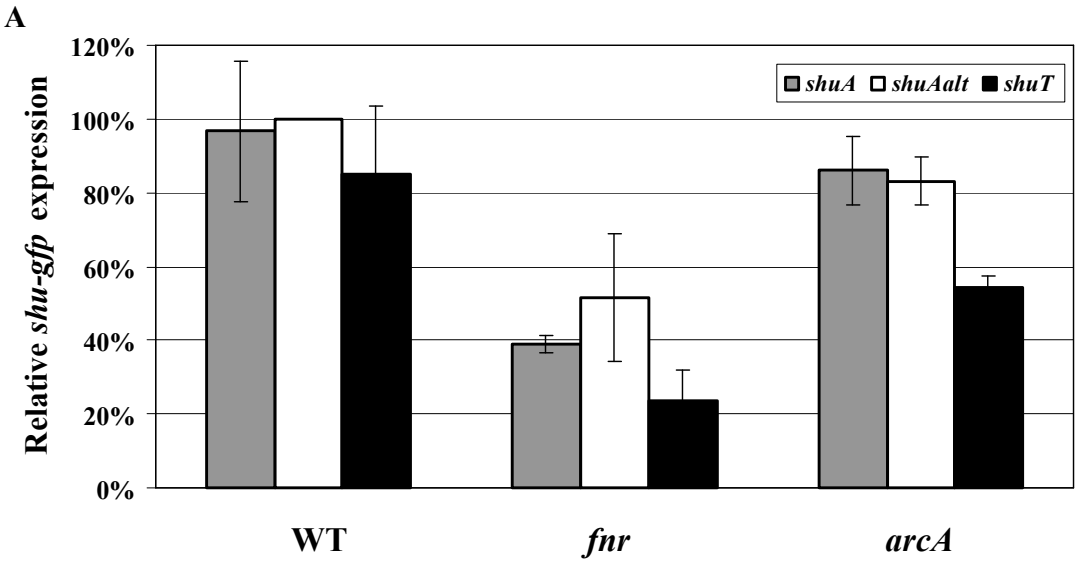
B. The native and altered promoter regions *fhuA* used in transcriptional *gfp*-fusions are depicted. The *fhuA* (pEG5) promoter contains a large portion of *mrcB,* while this gene and additional 5' sequence were removed from subsequent constructs. The putative ArcA box 1 (based on the ArcA box prediction in Fig. 28B), present in *fhuA*, has been deleted in *fhuAΔ*. *fhuAΔalt* contains altered bases in the ArcA box 2 region predicted to contain an ArcA box based on the Lynch and Lin motif (Lynch & Lin, 1996). A putative Fnr box is also present in *fhuA*, *fhuAΔ*, *fhuAΔalt*, and *fhuΔ2*. Bases matching the ArcA box or Fnr box consensus sequence are shown in black, and bases divergent from consensus are in gray. Lower case letters indicate bases not conserved in the ArcA or Fnr box weight matrix.

of the *shuA* and *shuT* promoters, but Fnr is required for full anaerobic expression from *shuT* and *shuA* (Fig. 23A). Three sequences resembling the Fnr box were found within the region between these divergent promoters (Figs. 23B and 23C). Several bases of the putative Fnr box that lies at the 5'end of the *shuA* promoter were altered in *shuAalt* (Fig. 23B); however, these changes did not appear to influence the regulation of this promoter (Fig. 23A). Whether the putative Fnr box that lies closer to the transcriptional start site of the *shuA* promoter is important for *shuA* activation by Fnr has not been determined.

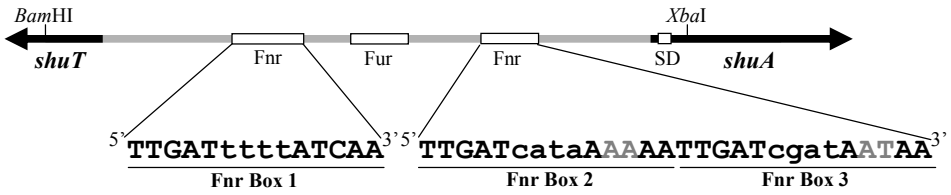
3.3.5. ArcA and Fnr Regulation of Additional Virulence Genes

Since several transcripts encoding the OspC family of proteins appeared to be increased in wild type *S. flexneri* relative to the *arcA fnr* mutant (Tables 11 and 12), their regulation was investigated further. Both OspC2 and OspC3 proteins are secreted by the TTSS and require the Spa15 chaperone for stability (Page *et al.*, 2002). The *ospC2*, *ospC3*, and *ospC4* genes are 96% identical (Buchrieser *et al.*, 2000), thus, oligonucleotide spots on the arrays could not distinguish among these transcripts. The *ospC4-C3* locus (s0007-6) contains two deletions that incorporate a frameshift mutation in the sequence, inactivating its gene products (Buchrieser *et al.*, 2000). Another *ospC3* sequence is present (s0122), and this promoter, as well as the *ospC2* promoter (s0066), was fused upstream of the *lacZ* gene in the pQF50 reporter plasmid to determine whether these genes were regulated by oxygen availability. β-galactosidase activities were measured during aerobic and anaerobic growth, and no significant difference in promoter activity was observed (Fig 24). Although the array data would have to be confirmed by

Figure 23. Anaerobic expression and organization of *shuA* and *shuT* promoters

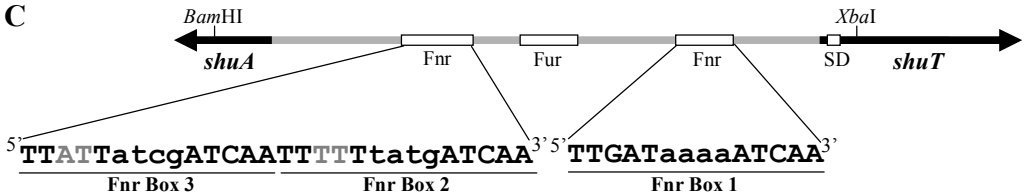


B



Promoter	Fnr Box 1	Fnr Box 2	Fnr Box 3
shuA	TTGATttttATCAA	TTGATcataAAAAA	TTGATcgatAATAA
shuAalt	CCGACttttCTCGG	TTGATcataAAAAA	TTGATcgatAATAA

C



Promoter	Fnr Box 1	Fnr Box 2	Fnr Box 3
shuT	TTGATaaaaATCAA	TTTTTtatgATCAA	TTATTatcgATCAA

Figure 23. Anaerobic expression and organization of *shuA* and *shuT* promoters

A. The relative *gfp* expression obtained for the *shuA* (pMB*shuA*), *shuAalt* (pMB*shuAalt* contains bases altered in one of the putative Fnr boxes), and *shuT* (pMB*shuT*) promoter fusions in the avirulent WT (SA101), *fnr* (MBF100W), and *arcA* (MBF200W) strains are shown, with the highest relative fluorescence value set to 100%. The averages from three independent experiments are shown. Error bars represent one standard deviation.

B. The native and altered promoter regions *shuA* used in transcriptional *gfp*-fusions are depicted. The *shuA* promoter contains the native promoter, while base changes in the first of three predicted Fnr boxes are introduced into *shuAalt*. Bases matching the Fnr box consensus sequence are shown in black, and bases divergent from consensus are in gray. Lower case letters indicate bases not conserved in the ArcA or Fnr box weight matrix.

C. The native *shuT* promoter region used in the p*shuT* transcriptional *gfp*-fusion is depicted. *shuT* contains 45 bp of the *shuA* and *shuT* ORFs and the entire intergenic region, including the three putative Fnr boxes. Bases matching the Fnr box consensus sequence are shown in black, and bases divergent from consensus are in gray. Lower case letters indicate bases not conserved in the ArcA or Fnr box weight matrix.

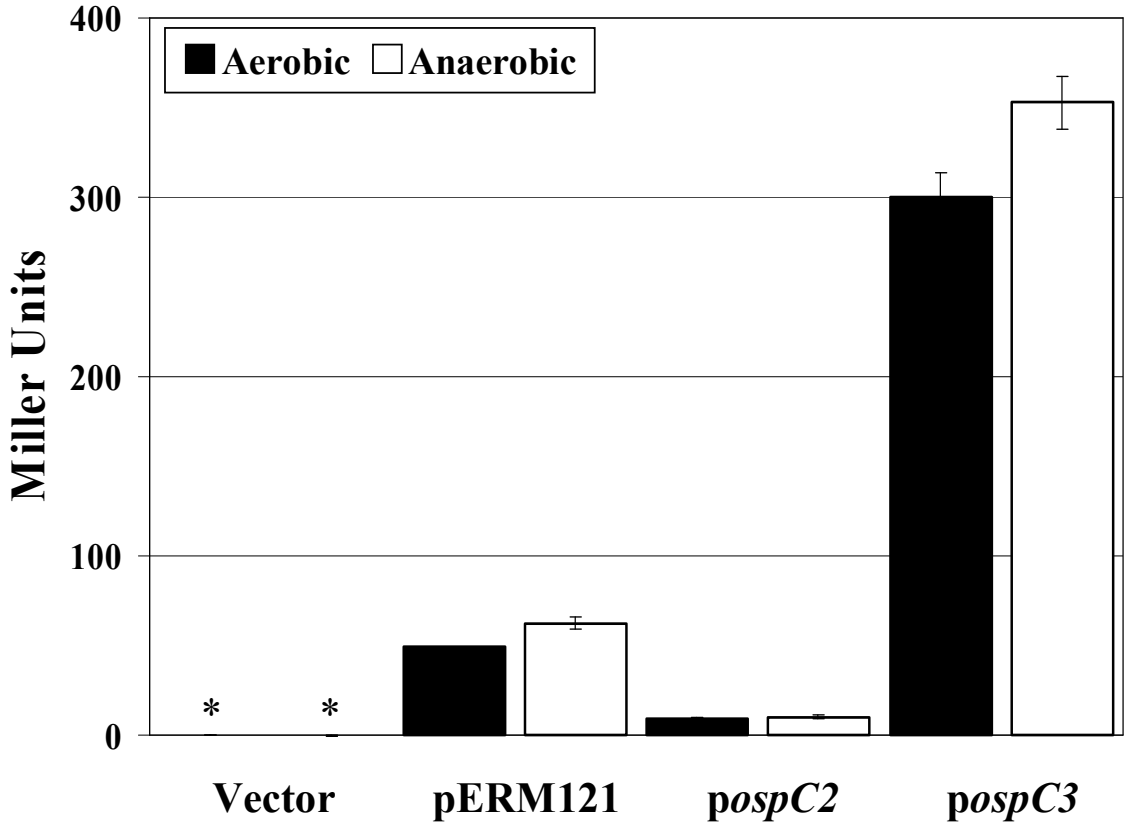


Figure 24. Expression of *ospC2-lacZ* and *ospC3-lacZ*

The relative β-galactosidase activities (in Miller units) obtained for the s0066 (*ospC2*) and s0122 (*ospC3*) promoter fusions of pMB*ospC2* and pMB*ospC3* in WT *S. flexneri* (SA100) during aerobic and anaerobic growth are shown. WT with plasmids pQF50 (vector) or pERM121 served as experimental controls. The averages from three independent experiments are shown. * indicates that the average β-galactosidase activity from pQF50 equaled zero. Error bars represent one standard deviation.

additional methods, perhaps transcript stability instead of activation is influenced by a factor that is anaerobically regulated, leading to their observed changes in microarrays.

4. ArcA Directly Regulates Expression of Iron Transporters and Fur

Since ArcA had not previously been shown to regulate iron uptake in any organism, experiments were undertaken to determine whether ArcA was acting directly on the *feo*, *iuc/iut*, and *sit* promoters. An additional possibility was that ArcA was affecting the expression of a regulator of these genes, and an obvious candidate was Fur. While microarray analysis did not indicate a two-fold or greater affect on *fur* transcription associated with the transition to anaerobiosis, additional assays were performed to investigate whether ArcA or Fnr influenced the expression of *fur*.

4.1. *FUR* TRANSCRIPTION IS REPRESSED BY ARCA

As an initial means of investigating whether ArcA or Fnr regulates *fur*, RT-PCR was performed with RNA isolated from anaerobically grown wild type *S. flexneri* and the *arcA, fnr*, and *arcA fnr* mutants. While there was no difference in the level of *fur* mRNA between the WT and *fnr* mutant, there was a significant increase of the *fur* transcript in strains lacking *arcA* (Fig. 25A), suggesting that *fur* transcription was repressed in an

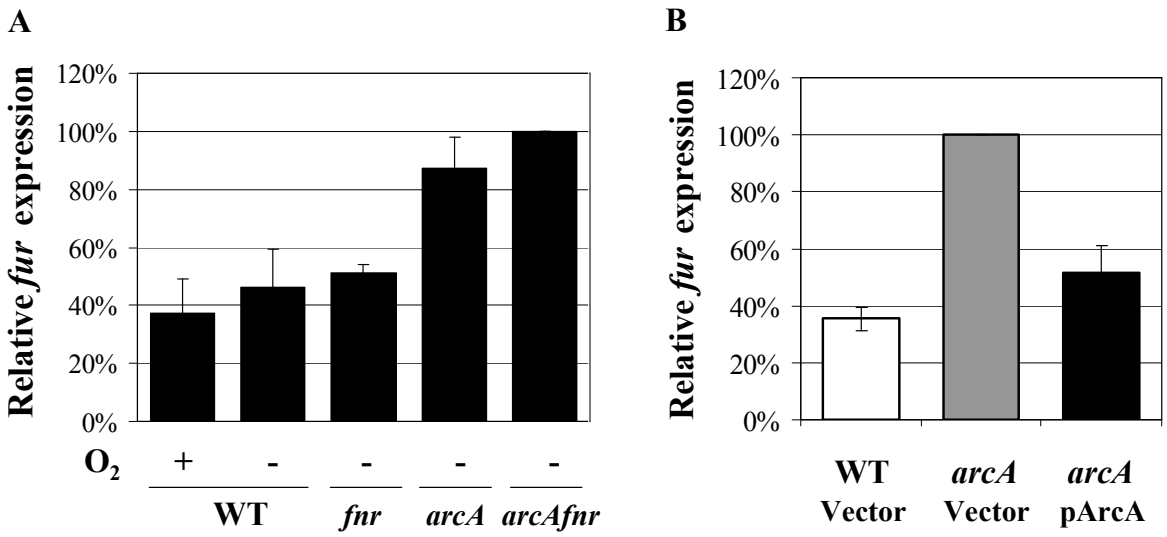


Figure 25. Real time RT-PCR indicates ArcA represses *S. flexneri fur* transcription

A. RNA was isolated from aerobically grown WT (SA100) and anaerobically grown WT (SA100), *fnr* (MBF100), *arcA* (MBF200), and *arcA fnr* (MBF300). The level of *fur* mRNA was determined by real time RT-PCR and normalized to levels of *rrsA*. The sample with the most abundant *fur* level was set to 100%. Data from three independent real time RT-PCR experiments are shown, with error bars indicating one standard deviation.

B. The level of *fur* mRNA was determined from WT (SA100) and *arcA* (MBF200) strains grown anaerobically with pCC1 vector or pArcA (pMB*arcA*ccQE) in the presence of 1μM IPTG by real time RT-PCR and normalized to levels of *rrsA*. The sample with the greatest *fur* level was set to 100%. Three independent experiments were averaged, with error bars indicating one standard deviation. The data in this figure were published previously by the author and used with permission from the publisher (Boulette & Payne, 2007).

ArcA-dependent manner. The role of ArcA was confirmed by restoring repression upon *arcA* expression from a plasmid (Fig. 25B). To ensure that ArcA was produced at wild type levels from this construct, an immunoblot with antiserum raised against ArcA was performed (Fig. 26). Strains were grown in the same manner used for RNA isolation for RT-PCR analysis except that medium contained differing concentrations of IPTG. ArcA was detected in cultures of MBF200/pMB*arcA*ccQE in the absence of IPTG induction, suggesting that there is leaky expression from this promoter. The amount of ArcA was approximately equal to wild type with 1 μM IPTG. In agreement, growth curves also demonstrated that the presence of 1 nM-1 μM IPTG complemented the growth defect of the *arcA* mutant (Fig. 16), allowing similar growth of SA100/pCC1 and MBF200/pMB*arcA*ccQE (data not shown). *fur* repression was restored in the *arcA* strain by inducing *arcA* expression from a plasmid (Fig. 25B), which provided evidence that the repression was due to ArcA and not the result of a secondary mutation.

For additional confirmation that ArcA, but not Fnr, regulates *fur* promoter activity, *gfp*-reporter assays were performed. A *gfp*-reporter fusion to the *fur* promoter was constructed and the relative fluorescence of *gfp* was determined in the wild type, *arcA*, *fnr*, and *arcA fnr* mutant strains. The GFP level was elevated in the *arcA* mutant relative to wild type (Fig. 27), indicating that this promoter was negatively regulated by ArcA. By contrast, there was no difference in fluorescence between the wild type and *fnr* strains, suggesting Fnr is not involved in regulation of *fur* transcription.

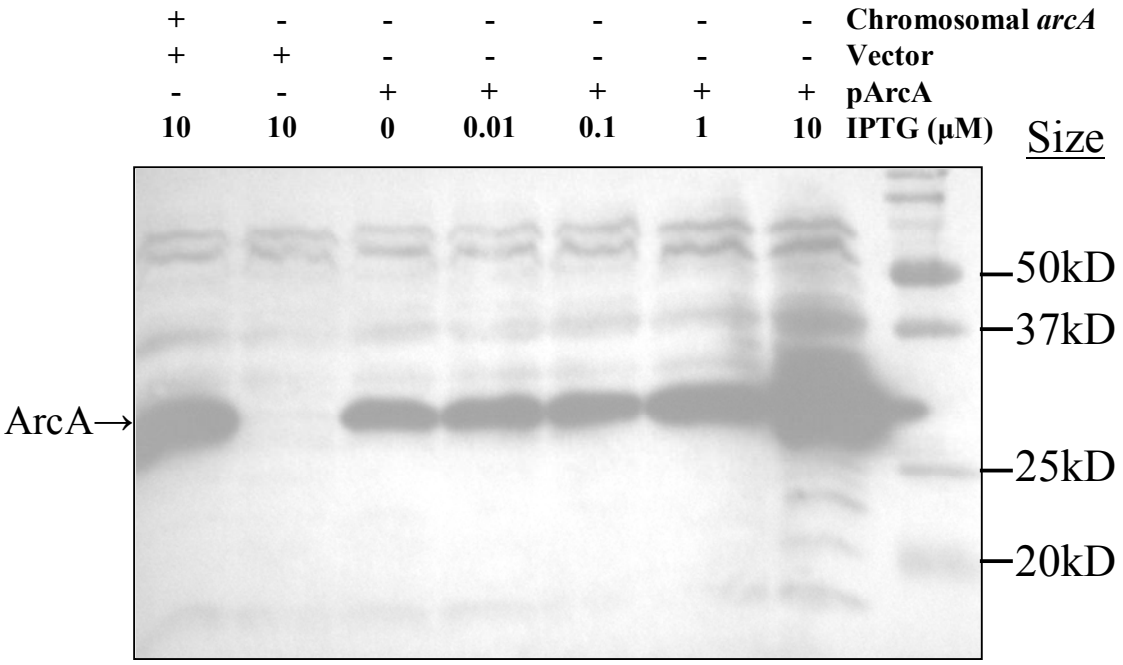


Figure 26. α-ArcA immunoblot to determine IPTG level for complementation

An α-ArcA immunoblot was performed on extracts from an IPTG titration with the *S. flexneri arcA/*pArcA strain to estimate the amount of IPTG necessary to restore ArcA levels. *arcA* (MBF200) containing pArcA (pMB*arcA*ccQE) was grown for two hours anaerobically in the presence of 10-fold serial dilutions of IPTG and compared to wild type (SA100) and *arcA* (MBF200) with pCC1 vector.

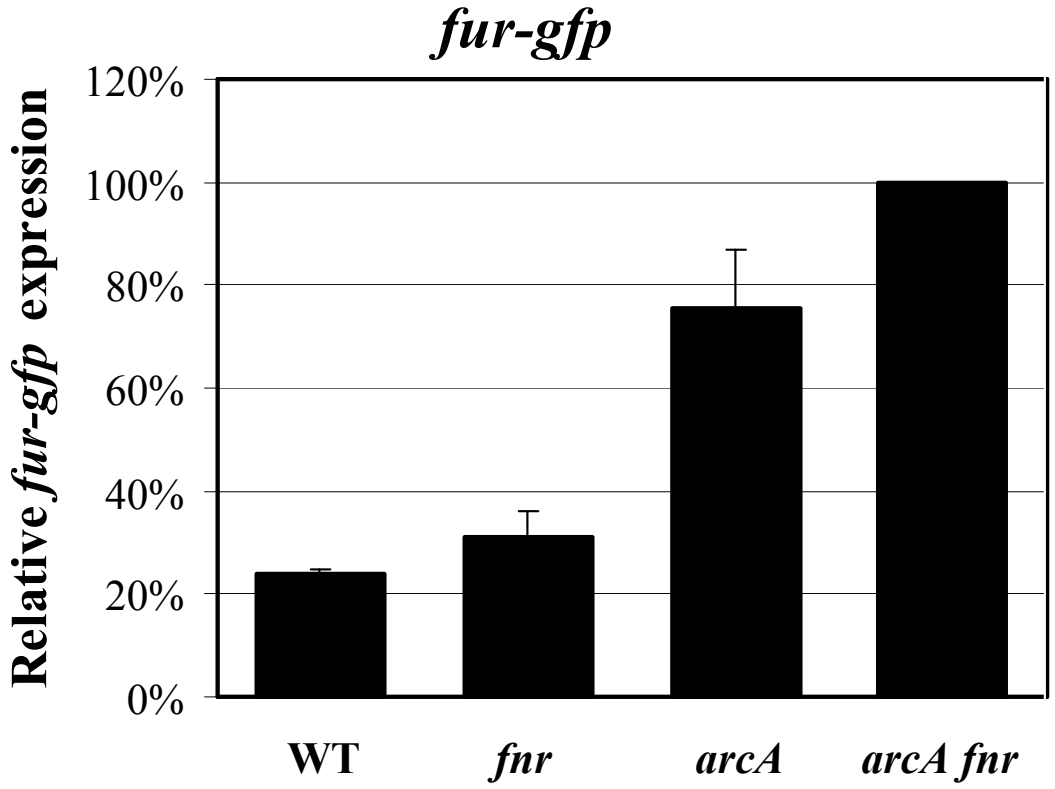


Figure 27. *fur-gfp* promoter activity is repressed by ArcA

The relative *gfp* expression obtained from the *fur* promoter of pMB*fur* in the avirulent WT (SA101), *fnr* (MBF100), *arcA* (MBF200) and *arcA fnr* (MBF300) strains are shown, with the highest relative fluorescence value set to 100%. The averages from three independent experiments are shown. Error bars represent one standard deviation. The data in this figure were published previously by the author and used with permission from the publisher (Boulette & Payne, 2007).

4.2. REDEFINING THE ARCA CONSENSUS

Although consensus sequences for the site recognized by ArcA have been reported in the literature (Drapal & Sawers, 1995; Favorov *et al.*, 2005; Liu & De Wulf, 2004; Lynch & Lin, 1996; McGuire *et al.*, 1999; Minagawa *et al.*, 1990), the optimal consensus for predicting ArcA binding is not known. Using parameters from one ArcA box prediction (Favorov *et al.*, 2005), and adding sequences from newly identified promoters for which ArcA regulation has been clearly demonstrated (Fig. 28A), we generated an ArcA binding consensus (Fig. 28B). This sequence was then used to identify putative ArcA binding sequences in the *feo* (Fig. 29), *iuc* (Fig. 30), and *fur* (Fig. 31) promoters. However, no sequence matching the ArcA consensus was identified in the *sit* promoter region, suggesting that the effect of ArcA on *sit* expression was indirect.

4.3. ALTERING PUTATIVE ARCA BOXES AFFECTS ARCA-DEPENDENT REGULATION

To determine whether the *feo* promoter region containing the putative ArcA box is important for ArcA regulation of *feo*, several bases were changed to the least common bases found at those positions in the weight matrix of the ArcA box consensus sequence (Fig. 29). The activity of the altered *feo* promoter (*feoAlt*) was similar in wild type and *arcA* strains, and was also lower than that of the native *feo* promoter in wild type (Fig. 32). These data suggested that ArcA did not stimulate expression from the altered *feo* promoter. The activity of the altered *feo* promoter in the *fnr* mutant was reduced to approximately half its level in the wild type strain (Fig. 32), which indicates Fnr binding

Figure 28. Putative ArcA binding sites and predicted ArcA regulatory motif

A.

Gene	Sequence identified in promoter	Reference for ArcA binding
E.c. aldA	TTCGGTGAAtaCATTGTTAA	(Pellicer *et al.*, 1999b)
E.c. cydA	ATAAGTTAActAAATGTAAA	(Lynch & Lin, 1996)
E.c. glcC-D	CTTGGTTAActCAATGTTAA	(Pellicer *et al.*, 1999a)
E.c. gltA-sdh	AAAAATTAAatAAATGTTGT	(Lynch & Lin, 1996)
E.c. icdA	TTTTGTTAAtgATTTGTAAT	(Chao *et al.*, 1997)
E.c. lldP	AATGGTTAActAAATGTTAT	(Lynch & Lin, 1996)
E.c. lpdA	TGTTGTTTAaaAATTGTTAA	(Cunningham *et al.*, 1998)
	AATTGTTAAcaATTTTGTAA	
	GTATTTTACaaAATTGTTAA	
	AATTGTTAAcaATTTTTAAA	
E.c. moe	ATAAATATCagAATTTTTAT	(Hasona *et al.*, 2001)
E.c. pflA	ATTAATAAAatAATTGTAAT	(Drapal & Sawers, 1995)
E.c. ptsG	ATTATTTTActCTGTGTAAT	(Jeong *et al.*, 2004)
E.c. soda	TATAGTTAAttAAATGATAT	(Tardat & Touati, 1993)
S.f feo	AGTTGTTAAtgAAGTGATAA	This study
S.f fur	AATTGCTACaaATTTGTAAC	This study
	AAAAGTTACaaATTTGTAGC	
S.f iuc	ATTATTGACatAATTGTTAT	This study

B.

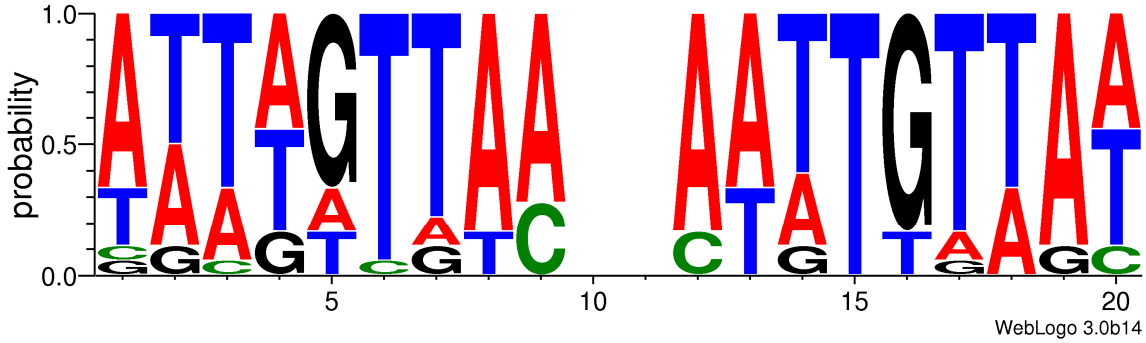


Figure 28. Putative ArcA binding sites and predicted ArcA regulatory motif

A. Promoter sequences of the genes listed were entered into the SeSiMCMC interface (http://favorov.imb.ac.ru/SeSiMCMC), and the algorithm reported a conserved weight matrix for ArcA sequence recognition. The putative ArcA box sequences found within these promoters are shown.
B. The sequence logo for the ArcA box is shown. It was obtained by entering the weighted matrix derived from multiple sequence alignment into the interface at http://weblogo.berkeley.edu. The data in this figure were published previously by the author and used with permission from the publisher (Boulette & Payne, 2007).

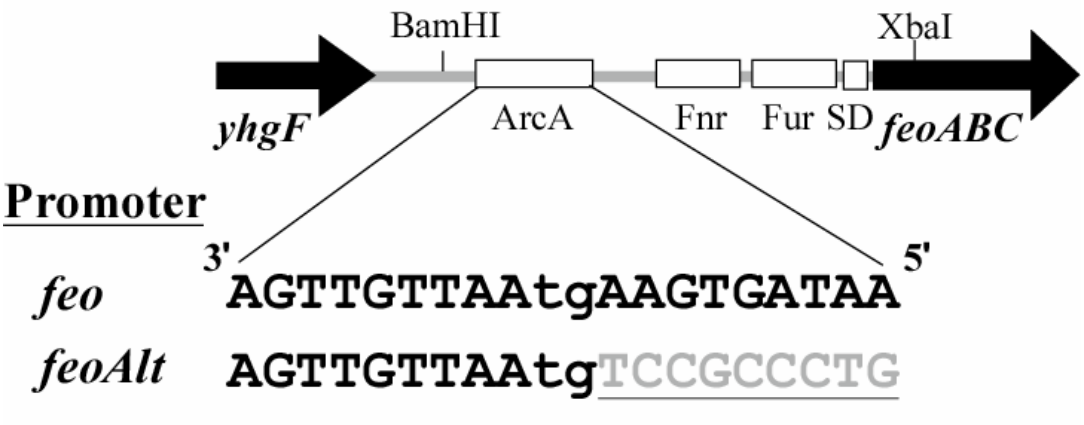


Figure 29. The *feo* promoter

The *S. flexneri feoABC* chromosomal region is depicted with promoter elements involved in *feo* regulation, including the putative ArcA box, Fnr box, and Fur box. The sequences of the putative ArcA box in the native and altered *feo* promoters are indicated below, with bases matching those of the putative ArcA box consensus sequence in black, bases divergent from consensus in gray, and bases changed in the altered promoter underlined. Lower case letters indicate bases not conserved in the ArcA box weight matrix. The data in this figure were published previously by the author and used with permission from the publisher (Boulette & Payne, 2007).

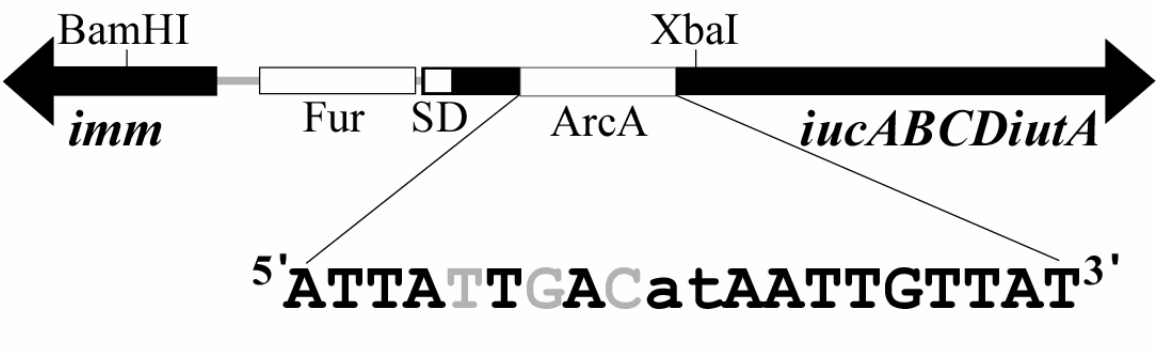
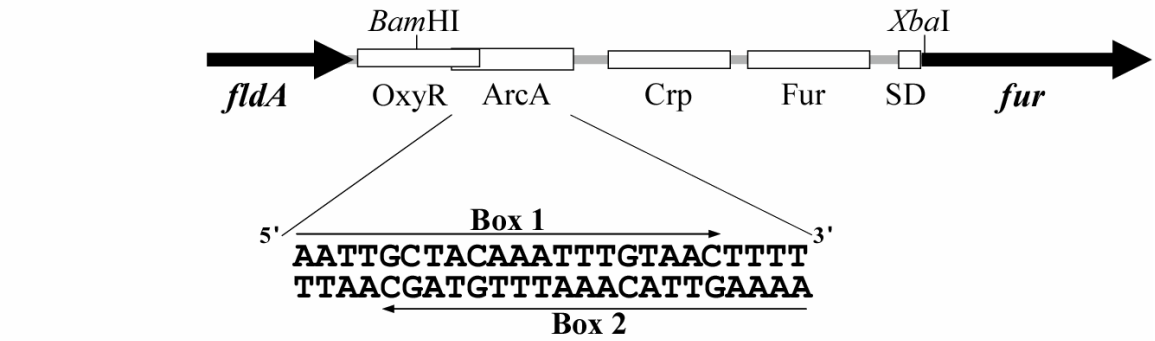


Figure 30. The *iuc/iut* promoter

The *S. flexneri iucABCD iutA* chromosomal region is depicted with promoter elements involved in *iuc* regulation, including the putative ArcA box and Fur box. The sequence of the putative ArcA box in *iuc* is shown below, with bases matching those of the ArcA box consensus sequence in black and bases divergent from consensus in gray. Lower case letters indicate bases not conserved in the ArcA box weight matrix. The data in this figure were published previously by the author and used with permission from the publisher (Boulette & Payne, 2007).



Promoter	Box 1	Box 2
fur	AATTGCTACaaATTTGTAAC	AAAAGTTACaaATTTGTAGC
furAlt	AATTGCGTTtcGTTTGTAAC	AAAAGTTACaaACGAAACGC

Figure 31. The *fur* promoter

The *S. flexneri fur* chromosomal region shows the relative position of promoter elements involved in *fur* regulation, including the putative ArcA boxes, OxyR box, Crp box, and Fur box. The two putative ArcA boxes are indicated by the arrows and their sequences are shown below. The bases in the native and altered *fur* promoters matching those of the putative ArcA box consensus sequence are shown in black. Bases not matching the consensus are in gray, and bases changed in the altered promoter underlined. Lower case letters indicate bases not conserved in the ArcA box weight matrix. The data in this figure were published previously by the author and used with permission from the publisher (Boulette & Payne, 2007).

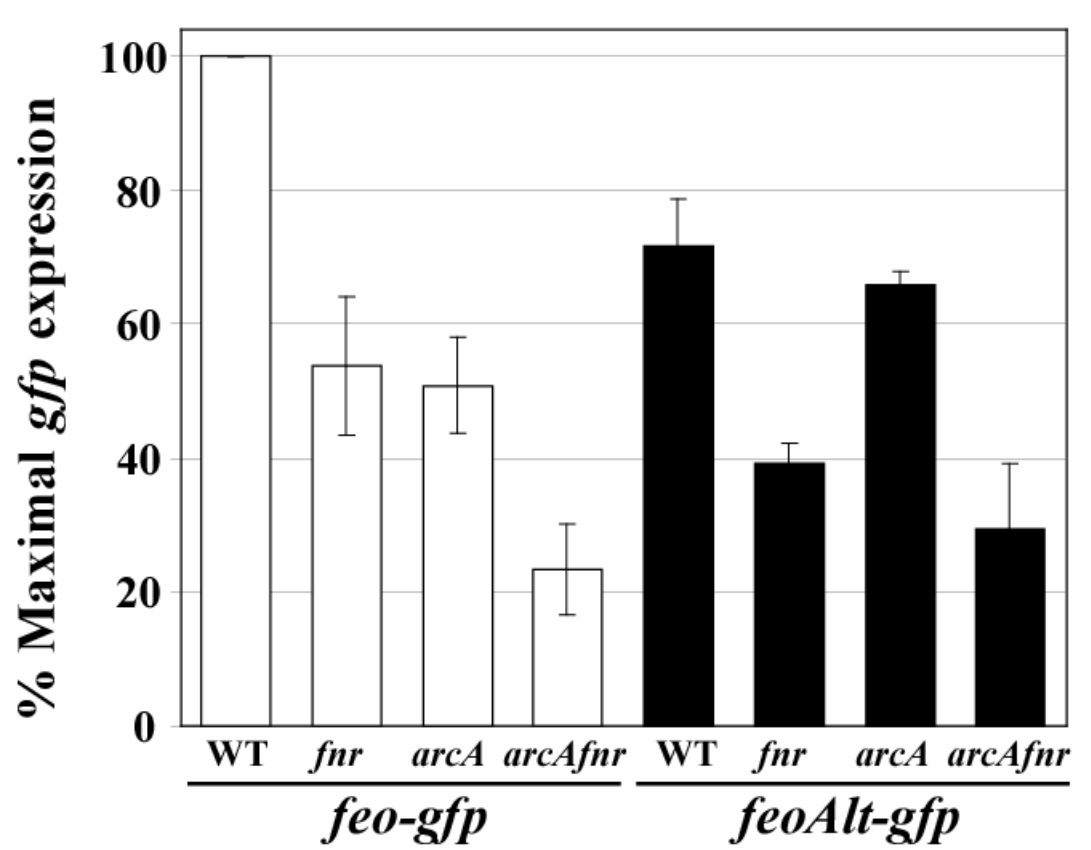


Figure 32. Anaerobic expression of native and altered *feo* promoter

The relative *gfp* expression obtained for *feo* and *feoAlt* promoter fusions of pMB*feo* and pMB*feoAlt* in the avirulent WT (SA101), *fnr* (MBF100W), *arcA* (MBF200W), and *arcA fnr* (MBF300W) strains are shown, with the highest relative fluorescence value set to 100%. The averages from three independent experiments are shown. Error bars represent one standard deviation. The data in this figure were published previously by the author and used with permission from the publisher (Boulette & Payne, 2007).

was not disrupted by the base changes that were introduced and suggests that ArcA induces anaerobic activation of *feo* at a site other than that induced by Fnr. Thus, the putative ArcA consensus sequence accurately predicted the region required for ArcA regulation of the *feo* promoter, and base alterations in the pMB*feoAlt* construct confirmed ArcA contributes to activation of the *feo* promoter.

Since the *fur* promoter appeared to be repressed by ArcA, I wanted to define the region of the *fur* promoter that was regulated by ArcA. Two putative ArcA boxes were found in the *fur* promoter, and a series of bases were then changed to the least common bases occurring at those positions in the weight matrix of the predicted ArcA box (Fig. 31, *furAlt*). The activity of the altered *fur* promoter was higher than that of the native *fur* promoter in the wild type strain (Fig. 33), and the activity of these promoters was similar in wild type and *fnr* strains. In addition, the difference between the amounts of fluorescence due to GFP in the *arcA* strain relative to the wild type was significantly decreased by the alterations made to the native promoter (Fig. 27 with 33). These results were consistent with ArcA repression of *fur* transcription and implicated the altered bases in contributing to ArcA binding at this promoter.

While the *iuc* promoter also contained a putative ArcA box, this sequence was found within the 5' coding sequence of *iucA* (Fig. 30). One could envision other reasons independent of altered ArcA regulation that could result in a change in *gfp* synthesis upon introducing base alterations in the ArcA binding sequence, for instance, if tRNAs corresponding to altered codons are more or less abundant in the cell than the original codon and would confer changes in the rate of translation. For this reason, the sequence

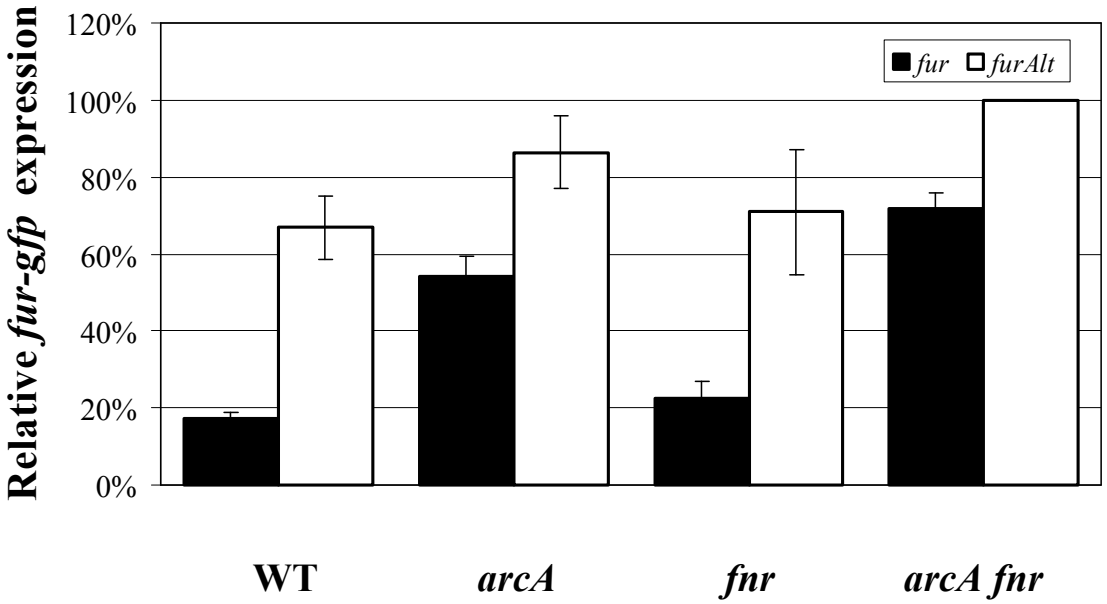


Figure 33. Altering the putative ArcA box relieves repression of *fur* transcription

The relative *gfp* expression obtained from *fur* and *furAlt* promoters in the avirulent WT (SA101), *arcA* (MBF200W), *fnr* (MBF100W), and *arcA fnr* (MBF300W) strains are shown, with the highest relative fluorescence value set to 100%. The averages from three independent experiments are shown. Error bars represent one standard deviation. The data in this figure were published previously by the author and used with permission from the publisher (Boulette & Payne, 2007).

resembling the putative ArcA box in the *iuc* promoter was not manipulated to investigate whether this site mediated ArcA repression of *iuc*.

4.4. ARCA BINDS PROMOTERS OF *FUR* AND IRON ACQUISITION GENES

Because transcription of *fur*, *iuc*, and *feo* was affected by the *arcA* mutation, and base alterations in putative ArcA boxes of the *fur* and *feo* promoters affected their activity in an ArcA-dependent manner, it was likely that ArcA directly regulated these genes. To assess whether there was a physical association of ArcA with the *feo*, *iuc*, and *fur* promoters, α-ArcA antibody super-shift assays were performed (Fig. 34). The *lld* (*lct*) promoter, for which ArcA binding has been demonstrated previously (Lynch & Lin, 1996), was used as a positive control. Incubation of the *lld*, *iuc*, *feo*, or *fur* promoters with an extract containing ArcA and α-ArcA antibody slowed electrophoretic mobility (Fig. 34, +ArcA lanes). Incubation of the probes with extract from the *arcA* mutant did not affect mobility of the radio-labeled probe (Fig. 34, -ArcA lanes). The altered *feo* and *fur* probes were also tested, and as predicted from *gfp* expression results (Fig. 32 and 33), the altered promoters showed reduced α-ArcA-dependent super-shifting (Fig. 34, compare *feo* with *feoAlt* and *fur* with *furAlt*). Some binding of ArcA to the altered probes was still observed, particularly with *furAlt*. Therefore, the amount of probe in the super-shifted bands was quantified by measuring radioactivity. The mean counts in the super-shifted regions were 3910 for the native *feo* probe compared with 1511 for *feoAlt* and 1918 for the native *fur* probe compared with 1607 for *furAlt*. Because there are regions with homology to the consensus ArcA box on both DNA strands of the *fur* probe (Fig.

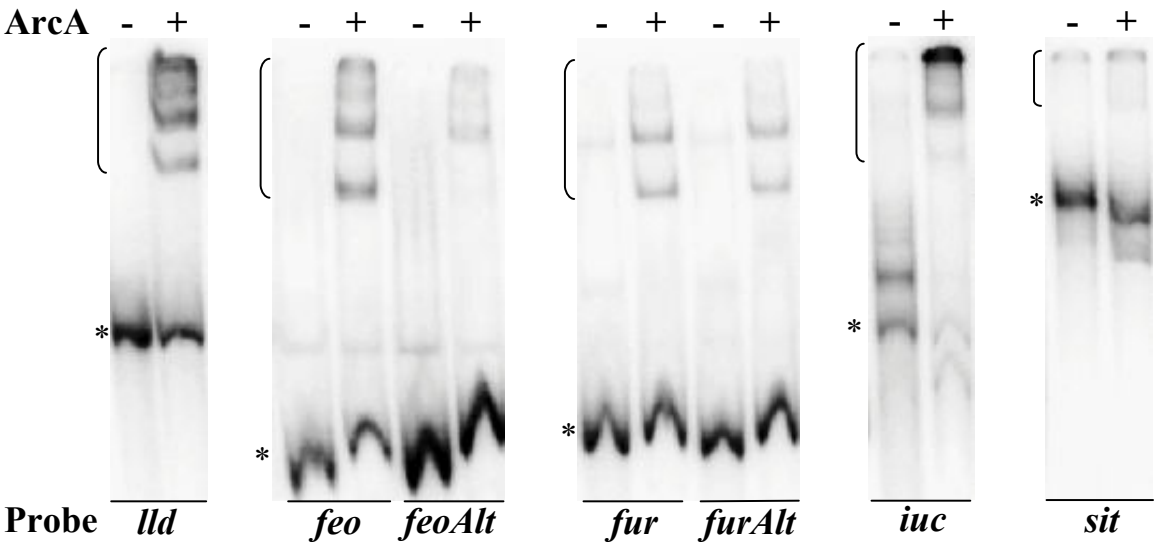


Figure 34. ArcA binds *feo*, *fur*, and *iuc* promoters

S. flexneri protein extracts prepared from the *arcA* strain containing either pCC1 vector ($ArcA^-$) or pMB*arcA*ccQE ($ArcA^+$) were incubated with a radio-labeled probe (*lld*, *feo*, *feoAlt*, *fur*, *furAlt*, *iuc*, or *sit*) and α-ArcA antibody. Samples were electrophoresed on a 5% polyacrylamide gel. Positions of probes are indicated with asterisks, and super-shifts are indicated with brackets. The data in this figure were published previously by the author and used with permission from the publisher (Boulette & Payne, 2007).

31), and the base changes introduced alter each site differently, there may be residual ArcA binding to one or both sites that could explain the abilities of *furAlt* to bind ArcA (Fig. 34, *furAlt*) and allow the altered promoter to be slightly repressed by ArcA (Fig. 33). ArcA did not bind the *sit* probe, indicated by the lack of super-shifting (Fig. 34, *sit*), which was in agreement with our failure to find an ArcA box sequence in the promoter. In contrast to the direct regulation of the *feo*, *fur*, and *iuc* promoters by ArcA, the ArcA regulation of *sit* may be indirect. These data demonstrate that ArcA directly binds the *feo*, *iuc*, and *fur* promoters, and that the altered bases of *feo* are within the region required for DNA recognition by ArcA. These data, along with the results from the GFP reporter studies using the altered *feo* and *fur* promoters, indicate that the consensus sequence used to identify the ArcA box may reflect the actual sequence recognized by ArcA.

IV. DISCUSSION

Studies on the growth of *Shigella spp.* in response to certain conditions encountered within the host have shed light on genes required for infection. However, there is scant information on the response of *Shigella spp.* to anaerobiosis, a condition present at the site of its invasion in the human. I have devised an anaerobic plaque assay and determined that it can be used to identify anaerobic virulence factors. I also determined the impact of the anaerobic environment on *S. flexneri* gene expression and showed that the anaerobic regulators ArcA and Fnr are important for the anaerobic regulation of iron acquisition and contribute to virulence in anaerobic environments.

1. Anaerobic Plaque Formation as a Virulence Model

Measures of *Shigella* virulence are typically performed in cell culture models by infecting epithelial monolayers and assaying adherence, invasion, intracellular growth, and intercellular spread of bacteria. Since these tissue culture models have typically been performed aerobically, and the intestinal environment is likely to be anaerobic, I have developed a plaque assay model of *Shigella* pathogenesis to measure *Shigella* infection in an anaerobic system. The use of both models of infection enables the distinction of

factors affecting virulence in environments limited and abundant in oxygen and may be of value in screening mutants in *Shigella* and other enteric pathogens before proceeding to test potential vaccine strains in primates.

In addition to providing information regarding intracellular iron usage, the anaerobic plaque assay was integral to determining the effect of mutations in the anaerobic regulators ArcA and Fnr on the virulence of *S. flexneri*. As these global transcriptional regulators are activated anaerobically and have been shown to affect virulence in other organisms, it was not surprising that these mutations prevented plaque formation under anaerobic but not aerobic conditions. ArcA and Fnr were not required for the expression of the virulence genes within the pathogenicity island found on the *Shigella* virulence plasmid (Tables 8 and 9). The *arcA fnr* mutant did exhibit reduced expression of genes involved in virulence compared to wild type (pp. 112-3 and Tables 10 and 11), yet this mutant had no defect in invasion (data not shown). Since ArcA and Fnr are pleotropic transcription factors with often overlapping functions, it is impossible to attribute the lack of infection by the *arcA fnr* mutant to only one pathway. Both ArcA and Fnr do, however, regulate iron acquisition on more than one level, and given the importance of iron acquisition in *S. flexneri* virulence, aberrant iron uptake in the *arcA fnr* double mutant is at least one cause for its inability to form plaques under anaerobic conditions.

2. Iron Acquisition in the Host

S. flexneri iron acquisition mutants are attenuated in plaque formation (Runyen-Janecky *et al.*, 2003). The iron-responsive transcriptional regulator, Fur, that regulates the expression of the iron transporters was also shown to be important for *S. flexneri* virulence. While no difference was observed in response to host oxygen availability, both mutation and over-expression of *fur* led to defective plaque formation by *S. flexneri* (Fig. 15). The reduction in numbers of plaques formed by the *fur* mutant was restored by mutation of *ryhB* in this strain (Payne *et al.*, 2006). Since *ryhB* over-expression reduces *virB* mRNA levels in *S. dysenteriae* and is responsible for decreased epithelial cell invasion (Murphy & Payne, 2007), the lack of *ryhB* repression in the *S. flexneri fur* mutant is predicted to account for its inefficiency in plaque formation. On the other hand, over-expression of *fur* in *S. flexneri* causes a titratable growth defect *in vitro* that appears to be exacerbated during intracellular growth (data not shown). These data demonstrate the importance of iron acquisition for the growth and virulence of *S. flexneri*.

During *S. flexneri* transit through host environments, it is likely that redox reactions impact the availability of free iron as changes in oxygen availability occur. Thus, it was important to determine the relative aerobic and anaerobic contributions of different iron acquisition systems during pathogenesis and whether the expression of the iron transporters was regulated by anaerobiosis. As a first step, I assayed iron transport mutants in aerobic and anaerobic plaque assays, and the results indicated that oxygen availability impacts the effectiveness of the different iron acquisition systems.

The Feo system plays an important role in *Shigella* virulence within the anaerobic epithelial environment. Since the Feo system alone supports wild type plaque formation during anoxia and *feo* is induced by ArcA and Fnr, it appears *S. flexneri* Feo is a Fe^{2+} transporter that is activated anaerobically in the epithelial environment. The inability of Feo to sustain aerobic plaque formation likely reflects its reduced expression when oxygen is abundant. The Feo iron transport system is integral to the intracellular survival and virulence of a number of pathogenic bacteria, including *L. pneumophila* (Cianciotto, 2007; Robey & Cianciotto, 2002), *C. jejuni* (Naikare *et al.*, 2006), *H. pylori* (Velayudhan *et al.*, 2000), and *S. enterica* serovar Typhimurium (Boyer *et al.*, 2002; Tsolis *et al.*, 1996). *E. coli* and *S. enterica* serovar Typhimurium *feoB* mutants are also attenuated in their ability to colonize the mouse intestine, which is likely on account of an inability to transport sufficient iron in this reduced oxygen environment (Stojiljkovic *et al.*, 1993; Tsolis *et al.*, 1996). There is also recent data that suggests Feo, in the absence of other iron transport systems, is sufficient for full virulence of *S. flexneri* in the mouse lung model of infection, whereas double mutants that only express either the Sit or aerobactin system are attenuated (E. Oaks, personal communication).

Not all host environments have limited oxygen availability; therefore, aerobically expressed iron transport systems are also predicted to be important for the virulence of *S. flexneri* and other pathogens. Under aerobic conditions the Sit system alone was sufficient for plaque formation similar to that of the wild type organism, which indicates the importance of Sit for aerobic iron acquisition. However, the presence of only the Sit system, which is also predicted to be capable of Fe^{2+} transport, led to the formation of small anaerobic plaques. Therefore, its activity or expression is reduced in anoxic environments such that it is unable to fulfill the demand for iron during oxygen

limitation. However, the Sit system also transports manganese (Kehres *et al.*, 2002), which correlates with increased survival of *S. flexneri* in macrophages and under oxidative stress (Runyen-Janecky *et al.*, 2006). Thus, Sit may be important to withstand oxidative bursts in macrophages in addition to its role in iron acquisition in both aerobic and anoxic conditions in the host.

Aerobactin is important for the transport of Fe^{3+} and its synthesis requires oxygen. Its expression was also repressed anaerobically (Figs. 12 and 19 and Table 6). *fhuF*, encoding a gene for the utilization of iron from hydroxamate siderophores including aerobactin, was elevated aerobically in *E. coli* (Kang *et al.*, 2005), and also appeared to be repressed anaerobically by ArcA in *S. flexneri* (Table 8 and Appendix C). Therefore, aerobic dependence of siderophore acquisition and usage may be conserved. Hence, it is not surprising that the *feo sit* mutant was unable to form plaques anaerobically. This mutant only allowed plaque formation in the presence of oxygen, and the size of the plaques was smaller than wild type. These data imply that siderophore-mediated iron acquisition is less significant than other mechanisms of iron transport for the intracellular growth of *S. flexneri* in the presence or absence of oxygen. This finding is in agreement with previous results by our laboratory that demonstrate the aerobactin locus is repressed during intracellular growth (Headley *et al.*, 1997). Additionally, the aerobactin system has been shown to be important for *Shigella* growth within extracellular tissues (Nassif *et al.*, 1987). Thus, a conservation of siderophore iron acquisition systems among *Shigella* and related species may be related to their role during certain aerobic, extracellular stages of pathogenesis.

3. Novel Targets Define a Role for ArcA in Regulation of Iron Acquisition

3.1. ARCA REGULATES FUR AND IRON ACQUISITION GENES

ArcA and Fnr are directly responsible for the anaerobic activation of *feo* transcription, while ArcA represses transcription of *iuc* and *fur* in *S. flexneri* under these conditions. The Fnr regulation of *feo* transcription was expected, since induction of the *feo* promoter under anaerobiosis was previously shown to be Fnr-dependent in *E. coli*, and the *S. flexneri feo* promoter sequence is identical to that of *E. coli feo*. This identity includes a site with similarity to the Fnr consensus sequence (Kammler *et al.*, 1993). However, ArcA regulation of the *feo* promoter has not been shown previously. Also novel is the direct regulation in *S. flexneri* of *fur* and *iuc* transcription by ArcA.

Although it was observed that expression of the *sit* operon was elevated aerobically in wild type but not the *arcA* mutant, its promoter did not appear to be directly regulated by either ArcA or Fnr. A recent study of transcriptional regulation of the *S. enterica* serovar Typhimurium *sit* operon has also demonstrated that its expression increased under aerobic conditions independently of ArcA and Fnr. Rather, Fur and MntR, in response to altered bioavailability of redox metal cofactors, contributed to the anaerobic reduction in *S. enterica* serovar Typhimurium *sit* transcription (Ikeda *et al.*, 2005). It was not determined, however, whether *fur* transcription was induced by anaerobiosis, which could lead to an increase in Fur repression of *sit* transcription. Since

an anaerobic induction of *fur* would lead to repression of iron transporters, and *feo* transcription was induced anaerobically by ArcA and Fnr, these transcription factors could preclude Fur repression of *feo*, while still permitting Fur repression of the less anaerobically-favorable aerobactin and Sit iron transporters. However, I show in *S. flexneri* that anaerobic induction of *fur* does not occur and that ArcA instead represses transcription of *fur* (Figs. 25 and 33).

My results show that ArcA directly regulates iron acquisition, which agrees with studies that indicate *E. coli* regulates *fur* and iron acquisition genes by anaerobiosis and ArcA (Covert *et al.*, 2004; Ikeda *et al.*, 2005; Kang *et al.*, 2005; Khullar *et al.*, 2003; Liu & De Wulf, 2004). In one of these studies (Liu & De Wulf, 2004), putative ArcA boxes were identified upstream of the *E. coli feo* and *fur* operons, as well as within the promoter of the *fepE* gene for Fe^{3+}-enterobactin (a siderophore in *E. coli* not synthesized or transported by *S. flexneri*) transport, but only the *fur* and *fepE* transcripts demonstrated significant changes in expression in the *arcA* mutant. My investigation provides direct evidence that ArcA represses *fur* and confirms ArcA involvement in the regulation of *feo* and siderophore biosynthesis and transport genes, further emphasizing the role for oxygen availability as a general signal for regulation of iron acquisition (Figs. 19 and 32-34).

3.2. ARCA TIES IRON UPTAKE TO CENTRAL METABOLISM

There are factors in addition to oxygen deprivation that result in ArcA activation (Georgellis *et al.*, 2001; Malpica *et al.*, 2004; Malpica *et al.*, 2006). When the respiratory dehydrogenases, reductases, or electron carriers are inactive because of an inadequate supply of cofactors such as iron or a lack of substrate availability, reduced quinones accumulate in the membrane (Fig. 35). These quinols lead to autophosphorylation of the ArcB sensor, which then becomes a kinase to activate ArcA. Phosphorylation of ArcA induces multimerization of ArcA dimers, and active oligomers bind specifically to regulatory elements of target genes (Jeon *et al.*, 2001; Toro-Roman *et al.*, 2005), although there are some data that suggest even unphosphorylated ArcA is able to bind DNA (Lynch & Lin, 1996; Perrenoud & Sauer, 2005). Upon anaerobiosis, the aerobic respiratory complexes are no longer functional. Under these conditions, ArcA helps the cell conserve energy and acquire ATP through substrate-level phosphorylation by down-regulating energetically costly TCA cycle and aerobic respiratory enzymes and by inducing fermentation genes and anaerobic respiratory complexes. Since many of the aerobic respiratory and TCA cycle enzymes contain iron cofactors, protein turnover would release bound iron. Additionally, the fermentation byproducts, which acidify the extracellular environment, may help solubilize ferric iron complexes. To the extent iron deficiency prevented productive respiration, increasing transport of iron would promote efficient energy production through electron transport and subsequent ATP synthesis. When the environment is devoid of molecular oxygen, iron is soluble, iron-sulfur clusters are stable, and excess iron is not toxic to cellular components. Therefore, it may be advantageous for the cell to generally de-repress iron transport, repress synthesis of unneeded siderophore biosynthetic and uptake complexes, and specifically induce the Feo ferrous iron transporter during anaerobic conditions. In addition to the de-repression of iron transport, it is likely that ArcA repression of *fur* would lead to an increase in

Figure 35. Model describing impact of ArcA on bacterial iron acquisition

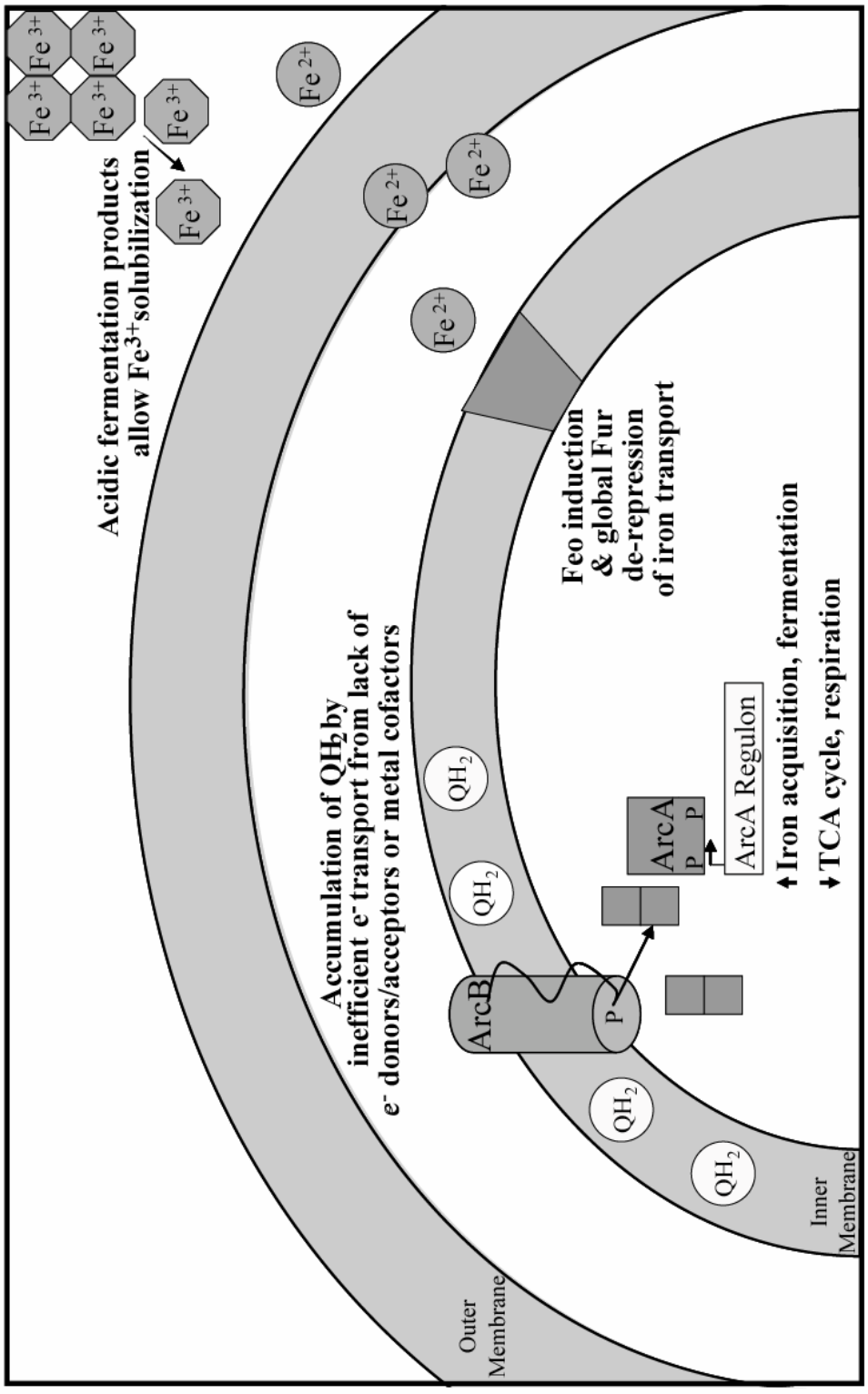


Figure 35. Model describing impact of ArcA on bacterial iron acquisition

Reduced quinones accumulate in the membrane due to inactive respiratory dehydrogenases, reductases, or electron carriers, causing autophosphorylation of the ArcB sensor and activation of ArcA. Phosphorylation of ArcA induces multimerization of ArcA dimers, and active oligomers bind specifically to promoter elements upstream of target genes. ArcA helps the cell conserve energy and acquire ATP through substrate-level phosphorylation by down-regulating energetically costly TCA cycle and aerobic respiratory enzymes and inducing fermentation and iron acquisition genes. Transcriptional repression of iron-containing enzymes by ArcA diverts iron to the most essential processes, while fermentation byproducts that acidify the extracellular environment may help solubilize ferric iron complexes. Additionally, *fur* repression by ArcA generally de-represses iron transport and concurrently increases RyhB-mediated effects on its target mRNAs that impact iron storage and cellular metabolism. ArcA also specifically represses aerobactin biosynthesis and uptake and induces the Feo ferrous iron transporter during anaerobic conditions.

RyhB. This would result in RyhB-mediated degradation of target mRNAs that include iron storage and oxidative stress genes. Interestingly, many of the targets of RyhB overlap with those of ArcA and Fnr, so an increase of RyhB may be a mechanism for additional fine-tuning of metabolic pathways during depletion of oxygen as well as iron.

4. Differences in Aerobic and Anaerobic Metabolic Gene Regulation in the Presence and Absence of Iron

By comparing the transcriptional profiles of *S. flexneri* genes regulated by oxygen availability in the presence and absence of iron (Tables 5 and 6), several conclusions can be reached about the effect of iron on cellular metabolism. In the presence of iron, several reductases and other anaerobic respiratory enzymes were elevated anaerobically, while in iron-limiting conditions only the anaerobic respiratory cytoplasmic nitrate reductases, hydrogenase maturation enzymes, and the molybdate metabolism regulator showed significant anaerobic elevation. Fermentation and F_1F_0 ATPase genes also were elevated to a greater extent anaerobically during iron restriction compared to iron abundance. Thus, anaerobic respiration may be the primary means to acquire energy in the presence of iron, while in the absence of iron, respiration is less effective and fermentation occurs until iron stores are replenished. This argument also agrees with the finding that the F_1F_0 ATPase shows a greater difference between aerobic and anaerobic conditions in the absence of iron. The expression F_1F_0 ATPase genes is reduced in response to slowing growth rate, which would be caused by a significant anaerobic

decrease in energy production during fermentation compared with anaerobic respiration. The F_1F_0 ATPase also functions in reverse under anaerobic conditions in the absence of respiration, extruding protons rather than using protons to generate ATP, which would contribute further to decreased growth. Genes involved in the TCA cycle showed significant changes in the presence and absence of iron; however, there were more TCA cycle genes that showed greater aerobic expression in the presence of iron. The *sodB* superoxide dismutase that is also involved in oxidative stress shows increased aerobic expression in the presence of iron. These differences are likely due to increased RyhB-mediated turnover of the *sdh-suc* and *sodB* transcripts during its de-repression in the absence of iron. Additionally, *suf* and *isc* operons for Fe-S cluster assembly show greater expression differences in the arrays without iron, which is in agreement with data suggesting that these genes are maximally expressed in oxidizing, iron-deplete conditions (Yeo *et al.*, 2006).

5. DNA Recognition by ArcA

The site required for recognition of DNA sequences by ArcA is not yet confirmed, yet numerous predictions have been made over the years (Drapal & Sawers, 1995; Favorov *et al.*, 2005; Liu & De Wulf, 2004; Lynch & Lin, 1996; McGuire *et al.*, 1999; Minagawa *et al.*, 1990). Comparing the putative ArcA boxes that have been reported, it is evident that requirements for recognition are becoming clearer (Fig. 36). It appears that the ArcA recognition sequence is a spaced repeat. Given the nature of the OmpR/PhoB family of winged-helix transcription factors, of which ArcA is a member,

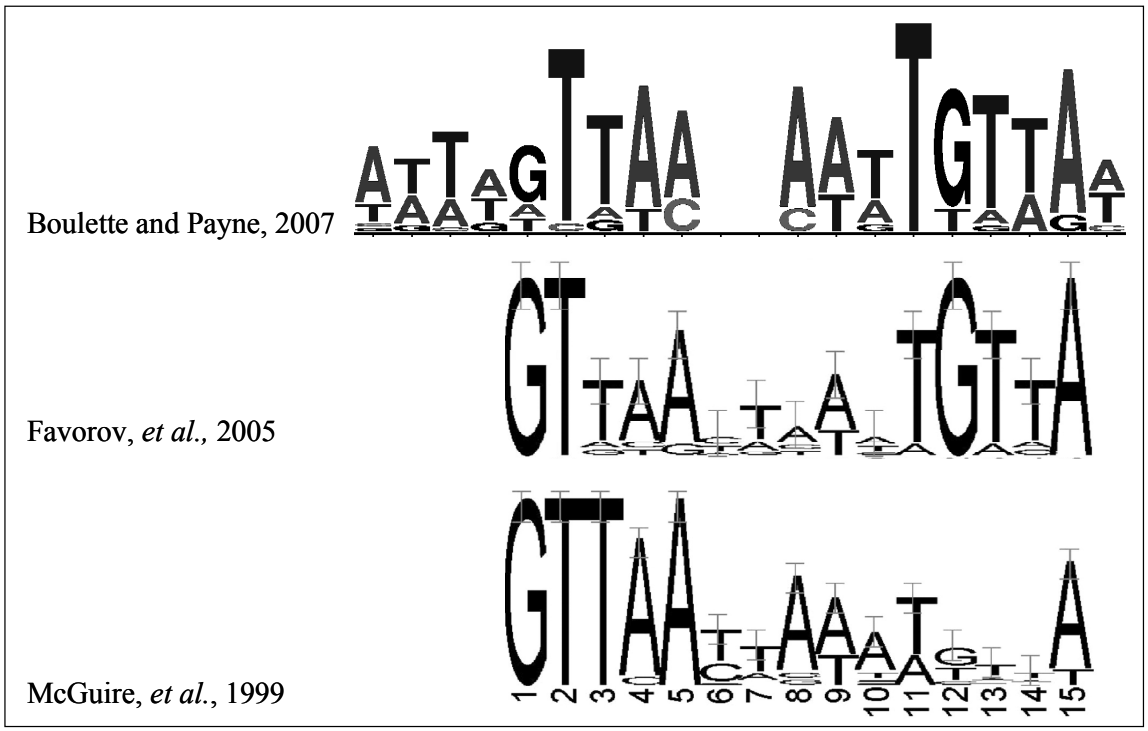


Figure 36. ArcA regulatory motifs

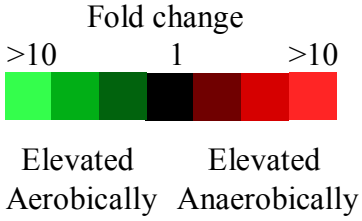
Comparisons of reported ArcA sequence logos show common features. Our prediction of the ArcA binding site shows a 20 base sequence containing a spaced repeat (sequence logo created at http://weblogo.berkeley.edu).

ArcA likely binds as a tetramer at DNA sequences that contain direct repeat half-sites (Toro-Roman *et al.*, 2005). Our putative ArcA binding site contains direct repeat half-sites, as repeats of 5 bases are present, possibly extending to 9-base repeats that are palindromic. More direct binding studies will further elucidate the size and nature of the sequence repeats.

APPENDICES

Appendix A: Microarrays demonstrate transcriptional changes of selected genes in the presence of iron in response to oxygen availability in *S. flexneri*.

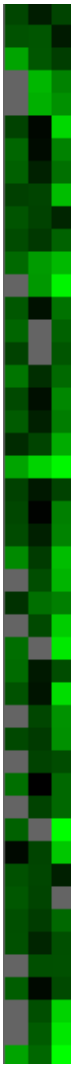
Bacteria were grown aerobically with added $FeSO_4$ to early logarithmic phase in a chemostat and harvested immediately before (aerobic) or 15 minutes after (anaerobic) oxygen depletion. RNA was then isolated, reverse transcribed, and labeled for use in microarray hybridizations. Average fold changes are derived from at least two experiments. Green spots indicate genes elevated aerobically, while red spots indicate greater anaerobic expression. Grey spots did not pass the spot filters. * indicates that standard deviations were only included for genes that met the quality control in at least three experiments.



Appendix A.1

Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Decreased Anaerobic Expression						
	B0001	*thrL*	Threonine biosynthesis	1.3-2.5	2.1	0.7
	B0002	*thrA*		1.6-2.9	2.3	0.7
	B0114	*aceE*	Pyruvate dehydrogenase	9.0-10.5	9.7	
	B0116	*lpdA*	Lipoamide dehydrogenase (NADH)	9.3-11.5	10.4	
	B0120	*speD*	Spermidine biosynthesis	1.6-3.4	2.4	0.9
	B0121	*speE*		1.6-3.4	2.6	0.9
	B0126	*yadF*	Putative carbonic anhydrase	2.7-8.9	6.2	3.2
	B0146	*sfsA*	Stimulates maltose utilization	2.1-2.8	2.4	
	B0156	*yadR*	Hypothetical	1.3-3.4	2.4	1.1
	B0178	*hlpA*	Periplasmic chaperone	1.2-2.7	2.2	0.8
	B0722	*sdhD*	Succinate dehydrogenase	3.9-4.7	4.3	
	B0724	*sdhB*		2.9-3.9	3.4	
	B0726	*sucA*	Succinyl transferase	2.5-3.1	2.8	
	B0728	*sucC*		7.7-7.8	7.8	
	B0729	*sucD*		3.6-7.9	5.7	
	B0755	*gpmA*	Phosphoglyceromutase	2.7-5.1	3.5	1.4
	B0810	*glnP*	Glutamine transport	1.6-28.9	12.4	14.6
	B0838	*ylif*	Putative transferase	1.8-4.1	2.6	1.2
	B0889	*lrp*	Leucine-responsive regulatory protein	1.7-2.8	2.2	0.6
	B0907	*serC*	3-phosphoserine aminotransferase	2.2-3.0	2.6	0.4
	B0929	*ompF*	Outer membrane proteins	1.6-2.7	2.1	0.6
	B0957	*ompA*		1.1-2.9	2.0	0.9
	B1136	*icdA*	Isocitrate dehydrogenase	2.4-10.4	6.4	
	B1243	*oppA*	Oligopeptide permease	3.5-8.0	5.8	2.3
	B1244	*oppB*		1.1-4.0	2.9	1.6
	B1247	*oppF*		1.6-3.2	2.6	0.9
	B1275	*cysB*	*cys* operon regulator	1.3-3.3	2.6	1.2
	B1324	*tpx*	Thiol peroxidase	6.6-8.2	7.4	
	B1402	*yi22_2*	Putative IS2 transposase	1.3-2.6	2.1	0.7
	B1482	*osmC*	Osmotically inducible protein	1.5-3.3	2.3	0.9
	B1656	*sodB*	Superoxide dismutase B	1.9-4.6	2.8	1.5
	B1713	*pheT*	Pheylalanine tRNA synthetase	1.0-2.4	1.9	0.8
	B1764	*selD*	Selenopeptide, possible formate oxidase component	1.6-3.6	2.5	1.0
	B1765	*ydjA*		2.0-7.0	4.5	
	B1836	*yebV*	Hypothetical	1.0-3.7	2.3	1.4
	B2181	*yejG*	Putative cytoplasmic protein	2.0-3.3	2.7	
	B2281	*nuoI*	NADH Dehydrogenase I	1.3-2.6	2.0	0.7
	B2285	*nuoE*		2.0-2.9	2.4	0.5
	B2286	*nuoC*		2.1-2.2	2.2	
	B2306	*hisP*	Amino acid transport	2.5-11.4	6.9	
	B2308	*hisQ*		3.0-11.1	5.8	4.6
	B2309	*hisJ*		1.2-21.9	9.1	11.2
	B2310	*argT*		4.3-54.6	29.4	
	B2412	*zipA*	Cell division protein	2.0-2.6	2.3	
	B2414	*cysK*	O-acetylserine sulfhydrylase	1.9-3.5	2.6	0.9
	B2463	*b2463*	Putative NADP-dependent multimodular enzyme	1.1-6.1	3.2	2.6

Appendix A.2

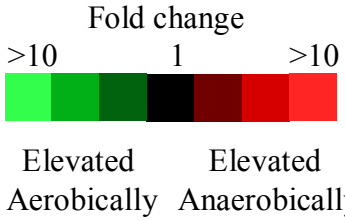
Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Decreased Anaerobic Expression						
	B2478	*dapA*	Dihydropicolinate synthase	1.4-2.1	1.9	0.4
	B2524	*yfhJ*	Fe-S cluster biosynthesis	1.3-2.5	2.1	0.6
	B2528	*iscA*		1.9-5.8	3.4	2.1
	B2529	*iscU*		4.0-7.0	5.5	
	B2530	*iscS*		4.6-6.5	5.6	
	B2597	*yfiA*	Putative σ^{54} modulator	1.1-10.0	4.4	4.9
	B2666	*b2666*	Putative YqaE family transport protein	1.1-4.0	2.7	1.5
	B2696	*csrA*	Carbon storage regulator	1.4-3.3	2.5	1.0
	B2741	*rpoS*	σ^S	2.0-7.5	3.9	3.2
	B2892	*recJ*	ssDNA exonuclease	1.5-2.5	2.0	0.5
	B2903	*gcvP*	Glycine cleavage system	1.8-2.8	2.3	0.5
	B2904	*gcvH*		3.1-7.2	5.2	2.1
	B2905	*gcvT*		5.1-13.4	9.2	
	B2924	*yggB*	Putative transport protein	1.2-3.4	2.4	1.1
	B3002	*yqhA*	Putative membrane-associated protein	2.8-2.9	2.9	
	B3024	*ygiW*	Putative outer membrane protein	2.0-2.5	2.3	
	B3097	*yqjC*	Unknown periplasmic and membrane proteins	1.6-3.5	2.7	1.0
	B3098	*yqjD*		1.2-4.2	2.5	1.5
	B3099	*yqjE*		1.4-3.8	2.6	1.2
	B3212	*gltB*	Putative inner membrane protein	1.6-6.3	3.3	2.6
	B3236	*mdh*	Malate dehydrogenase	5.7-13.9	9.7	4.1
	B3287	*def*	Peptide deformylase	1.3-2.0	1.8	0.4
	B3433	*asd*	Aspartate semialdehyde dehydrogenase	1.0-3.9	2.4	1.4
	B3460	*livJ*	Amino acid transport protein	1.4-4.1	2.6	1.4
	B3495	*uspA*	Universal stress protein	1.9-7.5	4.6	2.8
	B3553	*yiaE*	Putative 2-hydroxyacid dehydrogenase	2.3-6.8	4.6	
	B3829	*metE*	Methionine synthase	1.7-3.9	3.0	1.1
	B3830	*ysgA*	Putative carboxymethylenebutenolidase	2.2-8.9	5.5	
	B3908	*sodA*	Superoxide dismutase A	3.2-11.9	7.6	
	B4000	*hupA*	Histone-like DNA binding protein	1.3-3.1	2.2	0.9
	B4045	*yjbJ*	Putative cytoplasmic protein	1.3-10.5	4.7	5.1
	B4054	*tyrB*	Tyrosine aminotransferase	2.1-4.8	3.4	
	B4062	*soxS*	Positive oxidative stress regulator	1.9-4.6	2.9	1.5
	B4077	*gltP*	Glutamate transporter	2.0-2.2	2.1	
	B4172	*hfq*	RNA chaperone	1.0-3.3	2.4	1.2
	B4232	*fbp*	Fructose bis-phosphate	2.0-3.0	2.5	
	B4376	*osmY*	Hyperosmotic stress response protein	2.8-43.9	23.3	
	C0343	*c0343*	sRNA	1.1-8.3	3.8	3.9
	S0147	*mxiI*	TTSS proteins	1.4-2.4	2.0	0.5
	S0154	*mxiD*		2.1-2.5	2.3	
	SF0373	*s0373*	Hypothetical	1.9-3.0	2.4	0.6
	SF1323	*hns*	Nucleoid associated protein	1.5-2.4	2.1	0.5
	SF3178	*pic*	Protein involved in intestinal colonization	2.3-2.4	2.3	
	Z1134	*z1134*	IS3 putative transposase	1.1-2.7	2.0	0.8
	Z4883	*z4883*	HicA-like protein	2.3-9.5	5.9	
	Z5754	*ecnB*	Entericidin	2.7-11.7	7.2	
	Z5978	*z5978*	Putative inner membrane protein	3.0-23.3	10.7	11.0

Appendix A.3

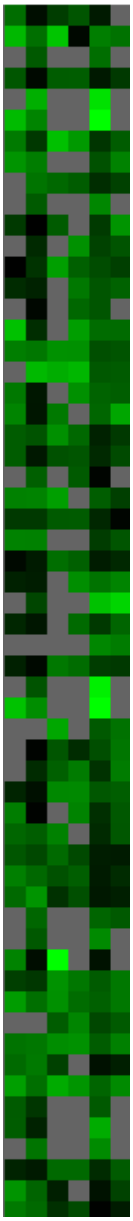
Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression						
	B0763	*modA*	Molybdate transport	7.7-9.5	8.6	
	B0764	*modB*		5.0-8.1	6.6	
	B0945	*pyrD*	Pyrimidine biosynthesis	1.1-9.4	4.5	4.4
	B1171	*b1171*	Hypothetical	1.6-2.9	2.2	0.6
	B1172	*b1172**		1.7-5.4	3.2	2.0
	B1223	*narK*	Nitrate Reductase	3.4-5.3	4.3	
	B1224	*narG*		9.2-39.2	27.8	16.2
	B1225	*narH*		4.0-18.4	13.2	8.0
	B1226	*narJ*		2.2-18.1	10.2	
	B1227	*narI*		3.7-14.5	9.1	
	B1541	*b1541*	Putative cytoplasmic protein	5.1-27.8	16.4	
	B1730	*b1730*	Hypothetical	2.1-2.8	2.4	
	B1751	*ydjY*	Hypothetical	1.1-3.8	2.9	1.6
	B1752	*ydjZ*		3.1-7.1	5.1	
	B2205	*napG*	Periplasmic nitrate reductase	1.8-4.3	3.1	1.3
	B2296	*ackA*	Acetate kinase	1.1-6.8	3.7	2.9
	B2343	*b2343*	Hypothetical	1.3-4.4	3.3	1.8
	B2434	*b2434*	Putative acyltransferase	2.4-3.3	2.8	
	B2552	*hmpA*	Redox flavoheme	1.2-3.2	2.2	1.0
	B2726	*hypA*	NiFe-hydrogenase maturation	1.5-6.4	3.5	2.6
	B2727	*hypB*		1.6-10.3	5.2	4.6
	B2729	*hypD*		3.3-4.4	3.8	0.6
	B2730	*hypE*		1.7-3.7	2.5	1.1
	B2850	*ygeF*	Hypothetical	1.5-3.8	2.5	1.2
	B2993	*hybD*	Hydrogenase synthesis	1.0-2.4	1.9	0.8
	B2995	*hybB*		1.1-7.5	4.8	3.3
	B2997	*b2997*		1.1-6.1	3.9	2.5
	B3158	*yhbU*	Putative collagenase	1.3-2.8	2.1	0.8
	B3262	*yhdJ*	Putative DNA methylase	1.6-3.1	2.2	0.8
	B3365	*nirB*	NADH-dependent nitrite reductase	5.4-57.0	31.2	
	B3510	*hdeA*	Acid responsive protein	2.9-3.8	3.3	0.5
	B3612	*yibO*	Putative phosphoglycerate mutase	2.6-7.4	4.3	2.7
	B3945	*gldA*	Glycerol dehydrogenase	4.6-7.5	5.7	1.6
	B4123	*dcuB*	Anaerobic C_4-dicarboxylate transporter	1.0-2.7	2.0	0.9
	B4154	*frdA*	Fumarate reductase	1.0-4.3	2.5	1.7
	B4244	*pyrI*	Pyrimidine biosynthesis	1.0-14.7	6.1	7.5
	B4245	*pyrB*		1.1-10.1	4.5	4.9
	B4299	*yjhI*	Putative transcriptional regulator	2.8-3.9	3.4	
	B4345	*mcrC*	Restriction enzyme	2.0-3.7	2.8	
	B4379	*yjjW*	Pyruvate formate lyase activating enzyme	2.5-2.7	2.6	
	C0362	*c0362*	sRNA	2.9-3.1	3.0	
	ECS1391	*ecs1391*	Bundle-forming pili-like protein	2.7-5.0	3.8	
	SF1245	*s1245**	Hypothetical, same as b1172	1.2-9.7	4.5	4.5
	Z0946	*z0946*	ATP-dependent dsDNA exonuclease	2.0-3.0	2.5	
	Z5889	*z5889*	O157-H7 putative protein	2.6-4.4	3.5	

Appendix B: Microarrays demonstrate transcriptional changes of selected genes in response to oxygen availability in *S. flexneri*

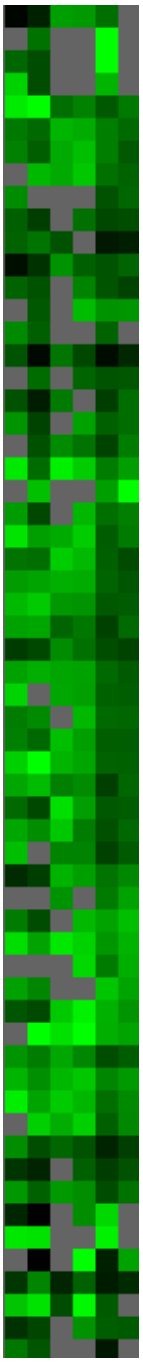
Bacteria were grown aerobically without added iron to early logarithmic phase in a chemostat and harvested immediately before (aerobic) or 15 minutes after (anaerobic) oxygen depletion. RNA was then isolated, reverse transcribed, and labeled for use in microarray hybridizations. Average fold changes are derived from at least two experiments. Green spots indicate genes elevated aerobically, while red spots indicate greater anaerobic expression. Grey spots did not pass the spot filters. * indicates that standard deviations were only included for genes that met the quality control in at least three experiments.



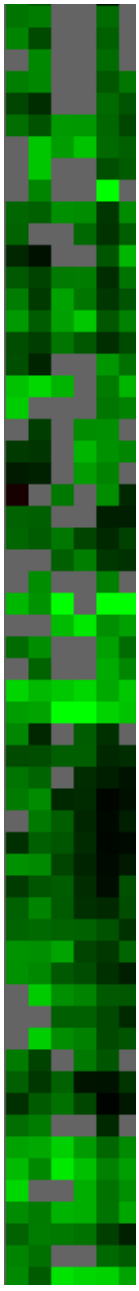
Appendix B.1

Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Decreased Anaerobic Expression						
	B0001	*thrL*	*thr* operon leader peptide	1.1-2.5	1.7	0.6
	B0014	*dnaK*	} Molecular chaperones	1.1-5.3	3.2	1.5
	B0015	*dnaJ*		2.1-2.5	2.3	
	B0026	*ileS*	Isoleucine tRNA synthetase	1.1-2.2	1.7	0.5
	B0114	*aceE*	Pyruvate dehydrogenase	4.4-6.7	5.5	
	B0116	*lpdA*	Lipoamide dehydrogenase (NADH)	3.0-9.1	5.7	3.1
	B0169	*rpsB*	Ribosomal protein	1.6-5.0	2.8	1.3
	B0170	*tsf*	Elongation factor	2.0-3.5	2.8	0.6
	B0220	*ykfE*	Hypothetical	1.5-2.7	2.1	0.5
	B0240	*crl*	Regulator of curli surface fibers	2.1-3.1	2.6	
	B0436	*tig*	Trigger factor chaperone for cell division	1.0-3.5	2.0	0.9
	B0439	*lon*	ATP-dependent protease	1.4-3.4	2.2	0.9
	B0453	*ybaY*	Hypothetical lipoprotein	1.0-3.8	2.0	1.0
	B0688	*pgm*	Phosphoglucomutase	1.3-2.7	2.0	0.6
	B0736	*ybgC*	Hypothetical	1.1-2.4	1.8	0.7
	B0740	*tolB*	} Envelope proteins for OM integrity	1.5-5.1	3.1	1.4
	B0741	*pal*		1.9-3.4	2.7	0.6
	B0756	*galM*	Galactose-1 epimerase	2.1-4.8	3.6	1.4
	B0767	*ybhE*	Putative isomerase	1.2-3.4	2.4	0.8
	B0889	*lrp*	Leucine-responsive regulatory protein	1.2-4.1	2.7	1.0
	B0911	*rpsA*	Ribosomal protein	1.3-3.4	2.2	0.7
	B0930	*asnS*	Asparagine tRNA synthetase	1.2-2.2	1.8	0.4
	B1087	*yceF*	Putative septum inhibitor	1.0-2.2	1.8	0.7
	B1089	*rpmF*	Ribosomal protein	1.7-3.8	2.7	0.8
	B1093	*fabG*	} Fatty acid biosynthesis	1.0-2.2	1.7	0.4
	B1095	*fabF*		1.7-3.0	2.4	0.7
	B1101	*ptsG*	PTS EII^{glc}	1.1-2.5	1.6	0.6
	B1102	*fhuE*	Coprogen transport	1.3-3.3	2.3	1.0
	B1243	*oppA*	} Oligopeptide permease	1.8-6.0	4.3	2.2
	B1246	*oppD*		1.2-2.5	1.8	
	B1257	*yciE*	} Hypothetical	2.9-3.1	3.0	
	B1259	*yciG*		1.1-2.7	1.8	0.7
	B1276	*acnA*	Aconitase A	2.0-7.5	4.8	
	B1324	*tpx*	Thiol peroxidase	3.3-8.9	5.8	2.9
	B1450	*yncC*	Putative regulatory protein	2.1-4.1	3.0	1.0
	B1478	*adhP*	Alcohol dehydrogenase	1.0-2.7	1.8	0.6
	B1679	*sufE*	} Fe-S cluster biosynthesis	1.4-2.8	2.2	0.7
	B1680	*sufS*		1.1-3.2	2.2	0.9
	B1681	*sufD*		1.0-3.0	2.1	0.9
	B1682	*sufC*		1.4-3.0	2.2	0.6
	B1684	*sufA*		1.3-2.4	1.8	0.5
	B1717	*rpmI*	Ribosomal protein	1.3-2.9	2.0	0.6
	B1718	*infC*	Initiation factor	1.2-3.5	2.0	0.9
	B1764	*selD*	Selenopeptide, possible formate oxidase component	2.1-2.4	2.3	0.1
	B1765	*ydjA*	Putative oxidoreductase	2.1-3.2	2.6	
	B1820	*b1820*	Putative inner membrane protein	1.1-9.0	3.6	3.7
	B1823	*cspC*	Cold shock protein	1.6-3.4	2.4	0.7
	B1824	*yobF*	Putative cytoplasmic protein	2.3-3.7	2.9	0.6
	B1832	*yebR*	} Hypothetical	1.8-3.1	2.3	0.5
	B1836	*yebV*		2.2-3.4	2.9	0.5
	B1852	*zwf*	Glucose-6-phosphate dehydrogenase	1.2-2.8	1.9	0.7
	B2185	*rplY*	Ribosomal protein	2.3-4.2	3.3	0.7
	B2234	*nrdA*	Ribonucleotide reductase	1.6-2.2	2.0	0.3
	B2309	*hisJ*	Amino acid transport	1.3-4.3	2.6	1.5
	B2463	*maeB*	Putative NADP-dependent malic enzyme	2.7-3.2	2.9	
	B2477	*nlpB*	Lipoprotein	1.2-2.4	1.8	0.6
	B2479	*gcvR*	Glycine cleavage system regulator	1.2-3.5	2.0	0.9
	B2518	*ndk*	Nucleoside diphosphate kinase	1.0-2.8	1.8	0.7

Appendix B.2

Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Decreased Anaerobic Expression						
	B2528	*iscA*	} Fe-S cluster biosynthesis	1.0-3.8	2.5	1.3
	B2529	*iscU*		2.6-15.3	9.0	
	B2531	*iscR*		1.9-20.4	8.2	10.5
	B2551	*glyA*	Serine hydroxymethyltransferase	1.9-5.9	4.1	2.0
	B2606	*rplS*	Ribosomal protein	2.1-8.5	4.4	2.8
	B2607	*trmD*	tRNA methyltransferase	2.4-4.6	3.2	1.0
	B2608	*yfjA*	rRNA processing protein	2.1-5.0	3.2	1.1
	B2609	*rpsP*	Ribosomal protein	2.3-5.4	3.8	1.3
	B2659	*ygaT*	Hypothetical	2.0-3.3	2.5	0.7
	B2674	*nrdI*	Ribonucleotide reductase	1.7-2.6	2.1	0.4
	B2687	*luxS*	Autoinducer-2 synthesis	1.2-2.7	1.9	0.6
	B2741	*rpoS*	σS	1.1-3.5	2.1	0.8
	B2742	*nlpD*	Lipoprotein D	1.8-3.0	2.2	0.5
	B2924	*yggB*	Putative transport protein	2.1-4.9	3.5	1.1
	B2942	*metK*	Methionine metabolism	2.2-3.2	2.6	0.6
	B3037	*ygiB*	Hypothetical	1.1-2.7	1.7	0.6
	B3065	*rpsU*	} Ribosomal proteins	2.0-2.5	2.2	0.2
	B3165	*rpsO*		1.3-2.8	2.1	0.6
	B3185	*rpmA*		1.7-3.9	2.8	0.9
	B3186	*rplU*		1.8-3.2	2.5	0.6
	B3231	*rplM*		2.5-7.6	4.9	2.2
	B3236	*mdh*	Malate dehydrogenase	3.8-8.5	5.8	2.4
	B3294	*rplQ*	Ribosomal protein	1.8-4.7	3.2	
	B3295	*rpoA*	RNA polymerase α subunit	2.2-6.9	4.3	1.7
	B3296	*rpsD*	} Ribosomal proteins	1.9-5.6	3.3	1.5
	B3297	*rpsK*		2.1-4.4	3.5	1.0
	B3298	*rpsM*		2.1-5.5	3.6	1.4
	B3299	*rpmJ*		1.7-4.1	2.9	0.9
	B3300	*prlA*	SecY secretion protein	1.7-3.3	2.2	0.6
	B3301	*rplO*	} Ribosomal proteins	2.1-4.4	3.5	0.9
	B3302	*rpmD*		2.2-6.0	3.7	1.5
	B3304	*rplR*		2.4-4.7	3.1	1.0
	B3305	*rplF*		2.2-5.0	3.1	1.1
	B3306	*rpsH*		2.3-11.4	5.0	3.4
	B3307	*rpsN*		1.7-4.9	3.2	1.1
	B3308	*rplE*		1.8-6.6	3.2	1.8
	B3309	*rplX*		2.0-5.4	3.3	1.3
	B3310	*rplN*		1.7-5.0	3.0	1.3
	B3313	*rplP*		1.4-4.5	2.9	1.2
	B3314	*rpsC*		2.8-3.9	3.4	0.5
	B3315	*rplV*		1.9-5.0	3.7	1.3
	B3317	*rplB*		3.6-7.0	5.2	1.4
	B3318	*rplW*		2.8-6.2	4.4	1.7
	B3319	*rplD*		3.1-5.3	4.1	0.9
	B3320	*rplC*		1.9-7.7	4.2	2.2
	B3321	*rpsJ*		4.0-9.7	6.8	2.6
	B3339	*tufA*	Peptide elongation factor	1.9-4.1	2.9	0.8
	B3340	*fusA*	Elongation factor	3.1-5.4	4.1	0.9
	B3341	*rpsG*	} Ribosomal proteins	2.6-7.5	4.6	1.8
	B3342	*rpsL*		2.3-5.8	4.3	1.6
	B3357	*crp*	cAMP receptor protein	1.3-3.3	2.1	0.7
	B3432	*glgB*	Glycogen branching enzyme	1.3-2.2	1.8	0.4
	B3433	*asd*	Aspartate semialdehyde dehydrogenase	1.9-3.7	2.9	0.7
	B3460	*livJ*	Amino acid transport protein	1.0-6.2	3.0	2.4
	B3544	*dppA*	Periplasmic dipeptide binding protein	6.7-7.7	7.2	0.5
	B3570	*bax*	Putative ATP-binding protein	1.0-8.7	3.7	3.5
	B3611	*yibN*	Hypothetical	1.3-3.1	1.8	0.7
	B3636	*rpmG*	} Ribosomal proteins	1.9-8.2	4.9	2.8
	B3637	*rpmB*		1.5-3.4	2.2	0.7
	B3639	*dfp*	DNA/pantothenate flavoprotein	1.2-2.8	2.0	0.8

Appendix B.3

Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Decreased Anaerobic Expression						
	B3703	*rpmH*	Ribosomal protein	2.5-2.9	2.7	0.2
	B3712	*yieE*	Hypothetical	1.9-3.1	2.4	0.6
	B3731	*atpC*	F_1F_0 ATPase subunits	2.5-3.2	2.8	
	B3732	*atpD*		2.1-3.1	2.7	0.4
	B3733	*atpG*		1.5-2.4	1.9	0.4
	B3734	*atpA*		1.8-3.6	2.7	0.8
	B3735	*atpH*		2.3-5.3	3.8	1.5
	B3736	*atpF*		2.0-5.3	3.1	1.9
	B3908	*sodA*	Superoxide dismutase A	3.1-16.3	9.7	9.4
	B3980	*tufB*	Elongation factor	1.6-3.4	2.7	0.7
	B3982	*nusG*	Transcription antitermination factor	1.5-2.4	2.1	0.4
	B3984	*rplA*	Ribosomal proteins	1.2-4.7	2.3	1.6
	B3985	*rplJ*		1.5-2.9	2.3	0.6
	B3986	*rplL*		1.6-3.9	2.5	0.9
	B3987	*rpoB*	RNA polymerase β subunit	2.1-5.4	3.3	1.2
	B4000	*hupA*	Histone-like DNA binding protein	1.5-2.7	2.0	0.4
	B4111	*proP*	Proline permease	1.4-3.6	2.4	0.9
	B4142	*mopB*	GroEL	2.8-6.2	4.7	1.2
	B4200	*rpsF*	Ribosomal protein	2.7-5.7	3.8	1.6
	B4201	*priB*	Primosomal replication protein N	1.7-3.4	2.8	1.0
	B4202	*rpsR*	Ribosomal protein	1.5-4.6	2.9	1.3
	B4203	*rplI*		1.3-3.7	2.3	1.2
	B4217	*ytfK*	Hypothetical	1.2-3.3	2.2	1.0
	B4220	*ytfM*		1.3-2.3	1.8	0.6
	B4226	*ppa*	Inorganic pyrophosphatase	1.4-2.7	2.0	0.5
	B4243	*yjgF*	Putative translational inhibitor	1.5-2.9	2.1	0.6
	B4244	*pyrI*	Pyrimidine biosynthesis	2.9-3.3	3.1	
	B4376	*osmY*	Hyperosmotic stress response protein	3.4-28.8	13.4	10.3
	S4056	*iucA*	Aerobactin synthesis and transport	3.3-7.0	4.8	1.6
	S4055	*iucB*		2.5-3.9	3.0	0.7
	S4054	*iucC*		2.3-4.2	3.2	1.0
	S4053	*iucD*		4.2-6.1	5.3	0.7
	S4052	*iutA*		3.8-9.4	6.2	2.5
	S0012	*shET2-2*	Enterotoxin	1.4-3.1	2.0	0.8
	S0039	*parA*	Plasmid partitioning protein	1.4-2.2	1.8	0.3
	S0051	*virF*	Master regulator of TTSS	1.2-2.7	1.8	0.7
	S0088	*ospD3*	TTSS secreted effector	1.0-3.2	1.8	0.9
	S0131	*virB*	Transcriptional activator of TTSS	1.1-2.8	1.8	0.7
	S0150	*mxiL*	TTSS apparatus proteins	1.1-2.2	1.6	0.5
	S0151	*mxiM*		1.1-3.4	2.0	1.0
	S0156	*mxiA*		1.1-2.2	1.7	0.4
	S0157	*spa15*	TTSS effectors and chaperones	1.4-3.0	2.0	0.6
	S0158	*spa47*		1.5-2.4	2.1	0.3
	S0159	*spa13*		1.6-4.0	2.8	1.0
	S0160	*spa32*		1.3-3.4	2.2	0.9
	S0161	*spa33*		2.1-5.5	3.2	1.4
	S0163	*spa9*		1.4-2.2	1.9	0.4
	S0166	*spa-orf10*		1.9-5.7	3.2	1.5
	S0192	*icsA*	Actin assembly protein for intercellular spread	1.8-2.8	2.4	0.5
	S0225	*ccdB*	Similar to addiction module	1.2-2.3	1.7	0.5
	SF1323	*hns*	Nucleoid associated protein	1.0-2.6	1.7	0.6
	SF2216	*S2216*	Hypothetical	1.4-2.9	2.3	0.8
	S1964	*sitA*	Manganese and iron transport	2.4-5.7	3.9	1.1
	S1965	*sitB*		3.0-7.2	4.5	1.6
	S1966	*sitC*		2.6-6.1	4.1	1.5
	S1967	*sitD*		2.7-4.5	3.6	0.8
	T44	*t44*	sRNA	1.3-2.9	2.3	0.6
	TPKE11	*tpke11*	sRNA	2.1-3.0	2.5	0.4
	Z5978	*z5978*	Putative inner membrane protein	2.1-6.7	4.7	1.8

Appendix B.4

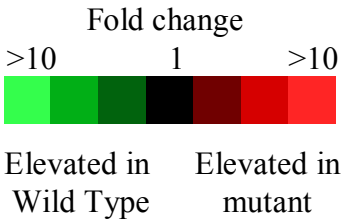
Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression						
	B0484	*ybaR*	Putative Cu-transporting ATPase	1.1-2.5	1.8	0.6
	B0557	*ybcU*	λBor homologue	2.7-2.3	2.1	0.4
	B0607	*ybdQ*	Hypothetical	1.6-3.0	2.3	0.6
	B0695	*kdpD*	Sensor for K^+ transport	1.6-2.1	1.8	0.2
	B0699	*ybfA*	Hypothetical	1.1-2.8	2.2	1.2
	B0733	*cydA*	Cytochrome D terminal oxidase subunit	2.2-2.2	1.6	0.5
	B0812	*dps*	Global starvation regulator/ iron storage protein	2.1-5.7	3.8	1.4
	B0820	*ybiT*	ATPase of putative ABC transporter	1.2-2.7	2.0	0.6
	B0856	*potH*	Putrescine permease	1.2-2.5	1.7	0.5
	B0876	*ybjD*	Hypothetical	1.0-2.4	1.8	0.6
	B0903	*pflB*	Formate acyltransferase	1.7-3.0	2.4	0.5
	B0905	*ycaO*	Hypothetical	1.5-2.3	1.9	0.4
	B1060	*yceP*	Hypothetical	1.7-11.4	6.7	4.3
	B1146	*b1146*	Hypothetical	2.3-5.0	3.7	
	B1171	*ymgD*	Hypothetical	1.3-2.9	1.9	0.8
	B1195	*ymgE*	Transglycosylase associated protein	1.8-4.6	2.8	1.5
	B1200	*ycgT*	Putative dihydroxyacetone kinase	1.6-4.3	2.5	1.2
	B1223	*narK*	Nitrate Reductase	2.2-30.5	12.2	12.8
	B1224	*narG*	Nitrate Reductase	2.8-32.1	15.5	14.4
	B1225	*narH*	Nitrate Reductase	1.9-15.4	8.8	6.1
	B1226	*narJ*	Nitrate Reductase	2.4-15.7	9.5	6.6
	B1227	*narI*	Nitrate Reductase	1.6-9.5	4.9	4.1
	B1241	*adhE*	Alcohol dehydrogenase	1.9-3.5	2.6	0.7
	B1283	*osmB*	Osmotically induced lipoprotein	1.4-2.7	2.2	0.5
	B1345	*intR*	Putative prophage integrase	1.1-3.4	2.2	1.1
	B1376	*ynaF*	Putative filament protein	1.5-4.9	3.1	1.2
	B1457	*ydcD*	Hypothetical	2.2-2.7	2.4	
	B1458	*b1458*	Hypothetical	1.4-2.4	2.0	0.5
	B1510	*ydeK*	Hypothetical	1.2-2.4	1.9	0.5
	B1519	*tam*	Putative trans-aconitate methylytransferase	2.1-6.3	3.7	2.2
	B1541	*b1541*	Putative cytoplasmic protein	5.6-36.9	18.9	14.0
	B1586	*ynfD*	Putative outer membrane protein	1.8-6.8	4.4	2.5
	B1593	*ynfK*	Putative dethiobiotin synthase	1.2-10.9	5.2	4.5
	B1774	*ydjJ*	Putative oxidoreductase	1.2-2.3	1.8	0.5
	B1906	*yecH*	Hypothetical	1.4-3.9	2.7	1.4
	B1947	*fliO*	Flagellar biosynthesis	1.1-2.9	1.9	0.7
	B2068	*alkA*	Methyladenine DNA glycosylase	1.2-2.2	1.7	0.4
	B2093	*gatB*	PTS $EIIb^{galactitol}$	1.4-2.4	1.9	0.4
	B2115	*molR_1*	Molybdate metabolism regulator	1.6-2.9	2.3	0.7
	B2168	*fruK*	Fructose utilization pathway	1.2-9.4	5.8	3.4
	B2169	*fruB*	Fructose utilization pathway	2.1-13.3	8.4	5.2
	B2343	*b2343*	Hypothetical	1.2-9.0	4.4	3.2
	B2452	*eutH*	Ethanolamine utilization	1.2-2.2	1.7	0.6
	B2579	*yfiD*	Putative formate acetyltransferase	2.0-4.8	3.4	
	B2726	*hypA*	NiFe-hydrogenase maturation	1.4-3.3	2.5	1.0
	B2727	*hypB*	NiFe-hydrogenase maturation	1.5-3.6	2.7	1.1
	B2728	*hypC*	NiFe-hydrogenase maturation	1.9-3.5	2.5	0.9

Appendix B.5

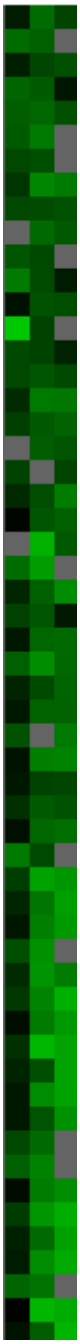
Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression						
	B2805	*fucR*	L-fucose operon activator	1.9-3.5	2.8	0.9
	B3007	*b3007*	Hypothetical	3.2-4.5	3.9	0.7
	B3108	*yhaM*		1.4-4.6	3.3	1.4
	B3109	*yhaN*		1.2-5.0	3.5	1.6
	B3110	*yhaO*		2.8-4.9	4.2	0.8
	B3194	*yrbE*		1.1-2.5	1.8	0.6
	B3234	*degQ*	Serine endoprotease	1.3-2.5	1.8	0.6
	B3408	*feoA*	Fe^{2+} transport	1.3-4.3	2.7	1.0
	B3409	*feoB*		1.7-3.1	2.2	0.6
	B3410	*feoC*		1.2-3.0	2.1	1.0
	B3505	*trs5_11*	IS5 transposase	1.4-4.3	2.5	1.3
	B3506	*slp*	Carbon starvation-induced outer membrane protein	1.2-3.0	1.9	0.6
	B3509	*hdeB*	Acid responsive proteins	1.3-3.0	2.3	0.7
	B3510	*hdeA*		2.0-4.1	3.1	0.8
	B3512	*yhiE*	Hypothetical	2.5-3.9	3.5	0.6
	B3517	*gadA*	Glutamate decarboxylase	1.5-3.8	2.6	1.0
	B3521	*yhjC*	Putative transcriptional regulator	1.4-5.6	3.0	2.3
	B3612	*yibO*	Putative phosphoglycerate mutase	1.8-4.2	2.8	1.0
	B3916	*pfkA*	Phosphofructokinase	1.3-5.4	3.1	1.8
	B4012	*yjaB*	Hypothetical acetyltransferase	1.3-2.2	1.7	0.4
	B4045	*yjbJ*	Putative cytoplasmic protein	1.4-2.5	2.0	0.4
	B4051	*qor*	Quinone oxidoreductase	1.4-3.3	2.2	0.7
	B4055	*aphA*	Acid phosphatase	1.1-2.3	1.6	0.5
	B4252	*yjgK*	Hypothetical	1.6-3.9	2.8	1.0
	B4313	*fimE*	Fimbriae regulatory recombinase	1.1-2.6	1.8	0.6
	B4341	*yjiS*	Hypothetical	1.1-2.5	1.9	0.5
	CSRB	*csrB*	sRNA regulator of CsrA	1.6-2.9	2.4	0.5
	ECS0235	*ecs0235*	Hypothetical	1.1-2.2	1.7	0.5
	ECS1519	*ecs1519*		1.0-2.2	1.7	0.5
	ECS4977	*ecs4977*		1.5-2.4	1.9	0.4
	GCVB	*gcvB*	sRNA regulator of glycine cleavage system	1.4-22.6	13.1	9.0
	S0135	*ipaC*	TTSS effector	1.4-2.3	1.9	0.4
	SF1370	*osmB*	Osmotically induced lipoprotein, same as b1283	1.0-3.0	2.1	0.8
	SPF	*spf*	sRNA	1.0-2.6	1.5	0.6
	SRAJ	*sraJ*	sRNA	1.2-2.3	1.7	0.5
	Z0961	*z0961*	Endopeptidase	1.3-2.2	1.8	0.5
	Z1141	*z1141*	Hypothetical	1.0-12.3	5.3	6.1
	Z1172	*terA*	Tellurium/colicin resistance, phage inhibition protein	1.2-2.3	1.7	0.5
	Z1375	*z1375*	Putative prophage tail protein	1.6-2.4	1.9	0.4
	Z1445	*z1445*	Unknown prophage proteins	1.3-2.2	1.7	0.4
	Z1460	*z1460*		1.2-2.4	1.8	0.6
	Z1905	*z1905*		1.1-2.3	1.7	0.6
	Z2141	*z2141*	Prophage tail proteins	1.6-2.9	2.0	0.5
	Z2353	*z2353*		1.4-2.4	1.8	0.4
	Z2989	*z2989*		2.1-3.3	2.7	
	Z4322	*z4322*	Hypothetical	1.5-2.4	2.0	0.4

Appendix C: Microarrays demonstrate transcriptional changes between *S. flexneri* wild type and the *arcA* mutant when grown anaerobically in iron deplete media

Bacteria were grown aerobically without added iron to early logarithmic phase in a chemostat and harvested 15 minutes after oxygen depletion. RNA was then isolated, reverse transcribed, and labeled for use in microarray hybridizations. Average fold changes are derived from at least two experiments. Green spots indicate genes elevated anaerobically in wild type, while red spots indicate greater anaerobic expression in the *arcA* mutant. Grey spots did not pass the spot filters. * indicates that standard deviations were only included for genes that met the quality control in at least three experiments.



Appendix C.1

Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression in Wild Type						
	B0131	*panD*	Aspartate decarboxylase		2.2	0.9
	B0180	*fabZ*	Fatty acid biosynthesis		2.9	
	B0185	*accA*	Acetyl Co-A carboxylase		1.9	0.5
	B0437	*clpP*	ClpAP serine protease subunit		2.3	0.9
	B0474	*adk*	Adenylate kinase		2.7	0.3
	B0523	*purE*	Purine biosynthesis		3.1	
	B0911	*rpsA*	Ribosomal proteins		2.2	
	B1089	*rpmF*	Ribosomal proteins		3.2	1.3
	B1093	*fabG*	Fatty acid biosynthesis		2.3	0.1
	B1101	*ptsG*	PTS EII^{glc}		2.8	
	B1133	*ycfB*	tRNA methyltransferase		2.3	
	B1172	*b1172*	Hypothetical		2.4	1.2
	B1334	*fnr*	Anaerobic regulator		1.9	0.7
	B1493	*gadB*	Acid responsive protein		5.2	
	B1539	*ydfG*	Putative oxidoreductase		1.9	0.4
	B1676	*pykF*	Pyruvate kinase		2.3	0.2
	B1716	*rplT*	Ribosomal protein		2.9	1.2
	B1823	*cspC*	Cold shock protein		2.5	0.5
	B1983	*yeeN*	Hypothetical		2.6	
	B2175	*spr*	Putative lipoprotein		2.0	
	B2415	*ptsH*	PTS $EIII^{glc}$		2.5	1.1
	B2518	*ndk*	Nucleoside diphosphate kinase		2.1	0.9
	B2551	*glyA*	Serine hydroxymethyltransferase		4.5	
	B2606	*rplS*	Ribosomal protein		4.2	
	B2608	*rimM*	rRNA processing protein		2.9	1.5
	B2830	*nudH*	Alarmone hydrolase/affects invasion		1.9	0.6
	B3065	*rpsU*	Ribosomal proteins		2.4	1.0
	B3165	*rpsO*	Ribosomal proteins		3.4	0.9
	B3175	*secG*	Secretion protein		2.2	0.7
	B3186	*rplU*	Ribosomal proteins		2.3	0.9
	B3230	*rpsI*	Ribosomal proteins		3.0	
	B3231	*rplM*	Ribosomal proteins		3.0	1.6
	B3251	*mreB*	Cytoskeletal septation/murein protein		1.8	0.4
	B3261	*fis*	Factor-for-inversion stimulation protein		2.2	0.5
	B3294	*rplQ*	Ribosomal proteins		2.5	1.1
	B3295	*rpoA*	Ribosomal proteins		2.9	
	B3296	*rpsD*	Ribosomal proteins		4.1	1.9
	B3297	*rpsK*	Ribosomal proteins		3.4	1.9
	B3298	*rpsM*	Ribosomal proteins		3.4	1.9
	B3299	*rpmJ*	Ribosomal proteins		3.5	
	B3300	*prlA*	SecY secretion protein		3.4	1.7
	B3301	*rplO*	Ribosomal proteins		4.1	2.3
	B3302	*rpmD*	Ribosomal proteins		3.3	1.9
	B3304	*rplR*	Ribosomal proteins		4.7	2.7
	B3305	*rplF*	Ribosomal proteins		3.1	2.2
	B3306	*rpsH*	Ribosomal proteins		3.8	2.2
	B3308	*rplE*	Ribosomal proteins		2.8	1.7
	B3310	*rplN*	Ribosomal proteins		2.6	
	B3311	*rpsQ*	Ribosomal proteins		3.3	
	B3313	*rplP*	Ribosomal proteins		3.1	1.7
	B3315	*rplV*	Ribosomal proteins		4.3	2.3
	B3316	*rpsS*	Ribosomal proteins		3.3	2.0
	B3317	*rplB*	Ribosomal proteins		3.9	2.4
	B3318	*rplW*	Ribosomal proteins		3.1	
	B3319	*rplD*	Ribosomal proteins		4.6	3.2
	B3321	*rpsJ*	Ribosomal proteins		3.5	2.8

Appendix C.2

Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression in Wild Type						
	B3340	*fusA*	Elongation factor	1.4-4.6	3.0	1.6
	B3342	*rpsL*	Ribosomal protein	2.0-3.7	2.8	
	B3409	*feoB*	Fe^{2+} transport	2.0-2.9	2.5	
	B3608	*gpsA*	L-glycerol 3-phosphate dehydrogenase	2.4-3.9	3.1	
	B3637	*rpmB*	Ribosomal protein	1.4-4.4	3.2	1.6
	B3639	*dfp*	DNA/pantothenate flavoprotein	1.4-2.8	2.2	0.7
	B3703	*rpmH*	Ribosomal protein	1.4-3.9	2.7	1.2
	B3980	*tufB*	Elongation factor	1.4-3.4	2.5	1.0
	B4005	*purD*	Purine biosynthesis	1.6-3.1	2.3	0.8
	B4147	*efp*	Elongation factor	2.1-2.2	2.1	
	B4201	*priB*	Primosomal replication protein N	2.3-8.2	5.1	2.9
	B4202	*rpsR*	Ribosomal proteins	3.0-5.6	4.4	1.3
	B4203	*rplI*	Ribosomal proteins	1.2-3.5	2.5	1.2
	FFS	*ffs*	4.5S RNA of SRP	3.0-3.0	3.0	
	SF1245	*s1245*	Hypothetical, same as b1172	2.2-6.2	3.5	2.3
	SF1323	*hns*	Nucleoid associated protein	1.8-2.2	2.0	0.2
	SPF	*spf*	sRNA	2.1-3.6	2.9	0.7
	SRAB	*sraB*	sRNA	2.3-4.8	3.5	
	T44	*t44*	sRNA	1.2-2.6	2.0	0.7
	Z5401	*z5401*	Putative cytoplasmic protein	1.6-4.8	3.1	1.6

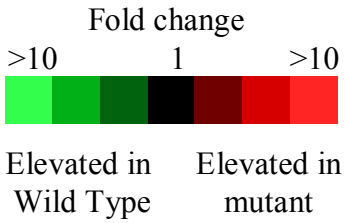
Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression in *arcA*						
	B0115	*aceF*	Pyruvate dehydrogenase	4.1-5.8	4.7	0.9
	B0116	*lpdA*	Lipoamide dehydrogenase (NADH)	8.8-9.7	9.2	0.4
	B0118	*acnB*	Aconitase B	2.7-4.2	3.4	
	B0353	*mhpT*	Putative transport protein	2.1-10.0	6.0	
	B0720	*gltA*	Citrate synthase	14.5-19.5	16.4	2.7
	B0721	*sdhC*	Succinate dehydrogenase	11.4-18.3	14.8	
	B0722	*sdhD*	Succinate dehydrogenase	11.6-80.1	40.0	35.7
	B0723	*sdhA*	Succinate dehydrogenase	12.3-38.3	26.2	13.1
	B0724	*sdhB*	Succinate dehydrogenase	8.3-24.0	15.7	7.9
	B0725	*b0725*	Hypothetical	19.4-22.8	21.1	
	B0727	*sucB*	Succinyl transferase	9.9-15.3	13.5	3.1
	B0728	*sucC*	Succinyl transferase	9.4-16.6	13.0	3.6
	B0729	*sucD*	Succinyl transferase	11.4-18.5	15.0	
	B0740	*tolB*	Periplasmic protein	2.0-3.1	2.5	0.6
	B0750	*nadA*	Quinolinate synthetase	2.1-3.7	2.8	0.9
	B0751	*pnuC*	Nucleoside transporter	2.1-2.9	2.5	0.4
	B0753	*b0753*	Putative homeobox protein	2.4-6.9	4.7	
	B0755	*gpmA*	Phosphoglyceromutase	1.6-2.9	2.2	0.6
	B0773	*ybhB*	Putative phospholipid binding protein	2.2-2.4	2.3	
	B0777	*bioC*	Biotin synthesis	2.1-2.1	2.1	
	B0810	*glnP*	Glutamine transport	3.2-3.3	3.3	
	B0811	*glnH*	Glutamine transport	9.9-11.4	10.6	
	B0812	*dps*	Global starvation regulator	1.7-7.1	4.1	2.7
	B0814	*ompX*	Outer membrane protein	1.5-4.5	2.7	1.6
	B0816	*b0816*	Hypothetical	1.8-4.2	3.3	1.3

Appendix C.3

Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression in *arcA*						
	B0838	*yliJ*	Putative transferase	2.0-2.3	2.2	
	B0880	*cspD*	Cold shock protein	1.3-2.7	2.0	0.7
	B0953	*rmf*	Ribosome modulation factor	1.2-2.6	2.1	0.8
	B1136	*icdA*	Isocitrate dehydrogenase	6.6-29.3	18.0	
	B1140	*intE*	Putative integrase	2.2-5.0	3.6	
	B1243	*oppA*	Oligopeptide permease	1.8-3.3	2.5	0.8
	B1276	*acnA*	Aconitase A	8.1-10.2	9.1	
	B1283	*osmB*	Osmotically induced lipoprotein	2.3-3.1	2.7	
	B1324	*tpx*	Thiol peroxidase	3.1-6.6	4.9	1.8
	B1336	*ydaH*	Putative transport protein	2.8-3.3	3.1	
	B1338	*ydaJ*	Probable hydrolase	1.1-2.8	2.2	1.0
	B1340	*ydaL*	Hypothetical	1.1-3.8	2.8	1.4
	B1342	*b1342*	Hypothetical	1.2-2.9	2.3	0.9
	B1344	*ydaO*	Hypothetical	1.0-3.8	2.7	1.5
	B1346	*ydaQ*	Hypothetical	1.0-3.2	2.4	1.2
	B1422	*b1422*	Hypothetical transcriptional regulator	2.6-3.6	3.1	
	B1632	*ydgQ*	Putative NADH:ubiquinone oxidoreductase	1.3-4.4	3.1	1.6
	B1743	*spy*	Periplasmic protein	1.6-2.9	2.2	0.7
	B1796	*yoaG*	Hypothetical	1.2-2.5	2.0	0.7
	B1800	*yeaU*	Putative tartrate dehydrogenase	1.4-2.9	2.3	0.8
	B1820	*yobD*	Putative inner membrane protein	2.3-2.9	2.6	
	B1890	*motA*	Motor protein	1.3-2.9	2.2	0.8
	B2133	*dld*	D-lactate dehydrogenase	1.9-2.5	2.3	0.3
	B2150	*mglB*	D-galactose periplasmic binding protein	2.3-2.4	2.4	
	B2181	*yejG*	Hypothetical	3.5-6.4	4.9	1.4
	B2309	*hisJ*	Histidine periplasmic binding protein	2.3-2.4	2.4	
	B2463	*maeB*	Malate dehydrogenase	2.2-2.3	2.3	
	B2490	*hyfJ*	Formate hydrogen lyase maturation	2.4-2.6	2.5	
	B2597	*yfiA*	Putative σ^{54} modulator	1.3-2.9	2.3	0.8
	B3073	*ygjG*	Probable ornithine aminotransferase	1.5-2.3	2.0	0.4
	B3160	*yhbW*	Possible mono-oxygenase	2.8-4.1	3.5	
	B3236	*mdh*	Malate dehydrogenase	3.2-5.7	4.5	
	B3430	*glgC*	Glucose-1-P adenylyltransferase	2.0-2.2	2.1	0.1
	B3530	*yhjL*	Putative oxidoreductase	2.6-2.7	2.7	
	B3603	*lldP*	Lactate permease	6.9-20.0	13.2	6.6
	B3605	*lldD*	Lactate permease	7.9-13.7	10.3	3.0
	B3942	*katG*	Catalase	6.9-8.2	7.6	0.7
	B3962	*udhA*	Pyridine nucleotide-disulphide oxidoreductase	3.1-4.9	3.8	1.0
	B3985	*rplJ*	Ribosomal subunit	1.3-2.9	2.2	0.8
	B4069	*acs*	Acetyl Co-A synthetase	6.1-11.6	8.8	2.8
	B4289	*fecC*	Fe^{3+}-citrate transport	2.2-2.3	2.3	
	B4365	*yjjQ*	Putative transcriptional regulator	1.4-5.8	3.5	2.2
	B4367	*fhuF*	Ferric iron reductase	1.2-3.8	2.9	1.5
	B4372	*holD*	DNA polymerase III Ψ subunit	1.3-4.8	2.8	1.8
	B4374	*yjjG*	Putative phosphatase	1.0-2.9	2.1	0.9
	B4376	*osmY*	Hyperosmotic stress response protein	3.0-15.8	11.0	7.0
	S0149	*mxiK*	T3SS protein	2.8-3.7	3.2	
	SF2649	*s2649*	Putative amino acid antiporter	1.9-3.3	2.4	0.7
	Z0312	*z0312*	Hypothetical	1.1-2.1	1.7	0.6
	Z1131	*z1131*	Hypothetical	2.3-3.0	2.6	
	Z3496	*z3496*	Hypothetical	2.0-2.3	2.2	0.2
	Z4857	*z4857*	Hypothetical	2.0-5.4	3.7	
	Z5978	*z5978*	Putative inner membrane protein	2.0-9.6	5.7	3.8

Appendix D: Microarrays demonstrate transcriptional changes between *S. flexneri* wild type and the *fnr* mutant when grown anaerobically in iron-rich media

Bacteria were grown aerobically with added $FeSO_4$ to early logarithmic phase in a chemostat and harvested 15 minutes after oxygen depletion. RNA was then isolated, reverse transcribed, and labeled for use in microarray hybridizations. Average fold changes are derived from at least two experiments. Green spots indicate genes elevated anaerobically in wild type, while red spots indicate greater anaerobic expression in the *fnr* mutant. Grey spots did not pass the spot filters. * indicates that standard deviations were only included for genes that met the quality control in at least three experiments.

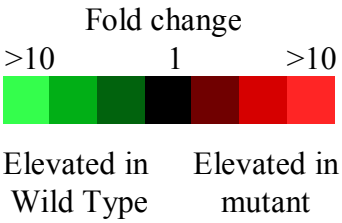


Appendix D.1

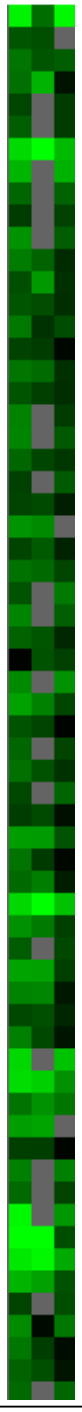
Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression in Wild Type						
	B1224	*narG*	Nitrate Reductase	2.1-4.9	3.5	
	B1225	*narH*		1.5-3.8	2.6	1.2
	B1227	*narI*		2.3-7.0	4.1	2.5
	B1836	*yebV*	Hypothetical	1.9-3.9	2.6	1.1
	B3186	*rplU*	Ribosomal protein	2.1-3.1	2.6	
	B3295	*rpo*	RNA polymerase α subunit	5.2-7.1	6.2	
	B3296	*rpsD*	Ribosomal protein	2.7-2.8	2.7	
	B3300	*prlA*	SecY secretion protein	2.2-11.8	5.5	5.4
	B3305	*rplF*	Ribosomal proteins	3.3-3.6	3.5	
	B3315	*rplV*		2.1-2.1	2.1	
	B3339	*tufA*	Peptide elongation factor	2.4-2.8	2.6	
	B3349	*slyD*	Peptidyl-prolyl isomerase	2.1-2.8	2.4	
	B3732	*atpD*	F_1F_0 ATPase subunit	2.3-3.1	2.7	
	B3985	*rplJ*	Ribosomal proteins	2.2-4.3	3.3	
	B3986	*rplL*		3.2-5.8	4.5	
	B4142	*mopB*	GroEL	3.3-10.2	6.5	3.5
	B4201	*priB*	Primosomal replication protein N	2.2-2.8	2.5	
	B4202	*rpsR*	Ribosomal protein	3.7-4.5	4.1	
	B4226	*ppa*	Inorganic pyrophosphatase	2.2-2.3	2.3	
	S0154	*mxiD*	TTSS protein	3.0-3.4	3.2	
	Z1377	*z1377*	Putative prophage tail protein	1.4-2.2	1.9	0.5
Increased Anaerobic Expression in *fnr*						
	B0674	*asnB*	Asparagine synthetase	4.2-5.3	4.8	
	B0734	*cydB*	Cytochrome d terminal oxidase	2.4-2.7	2.6	
	B1109	*ndh*	NADH dehydrogenase	1.8-3.7	2.9	1.0
	B1818	*manY*	Mannose PTS enzyme	2.3-3.2	2.7	
	B3521	*yhjC*	Putative transcriptional regulator	1.5-2.3	2.0	0.4
	B3549	*tag*	DNA glycosylase	2.3-3.1	2.7	
	S0006	*ospC3*	TTSS secreted effector	2.5-2.8	2.7	
	S0016	*sp0016*	Hypothetical receptor kinase	2.5-3.7	3.1	
	SSRA	*ssrA*	sRNA	2.1-3.7	2.6	0.9
	Z1336	*z1336*	Hypothetical prophage protein	1.2-4.4	2.6	1.7

Appendix E: Microarrays demonstrate transcriptional changes between *S. flexneri* wild type and the *arcA fnr* mutant when grown anaerobically in iron rich media

Bacteria were grown aerobically with added $FeSO_4$ to early logarithmic phase in a chemostat and harvested 15 minutes after oxygen depletion. RNA was then isolated, reverse transcribed, and labeled for use in microarray hybridizations. Average fold changes are derived from at least two experiments. Green spots indicate genes elevated anaerobically in wild type, while red spots indicate greater anaerobic expression in the *arcA fnr* mutant. Grey spots did not pass the spot filters. * indicates that standard deviations were only included for genes that met the quality control in at least three experiments.



Appendix E.1

Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression in Wild Type						
	B0014	*dnaK*	Molecular chaperone	3.8-20.2	14.3	9.2
	B0022	*insA_1*	IS1 protein	2.6-2.8	2.7	
	B0025	*ribF*	Putative regulator	2.8-3.1	3.4	0.7
	B0032	*carA*	Carbamoyl-phosphate synthetase	1.3-8.8	4.7	3.8
	B0094	*ftsA*	Cell division protein	2.1-2.3	2.2	
	B0131	*panD*	Aspartate decarboxylase	2.1-3.1	2.6	
	B0169	*rpsB*	Ribosomal protein	9.5-25.1	16.2	8.0
	B0170	*tsf*	Elongation factor	8.7-9.0	8.8	
	B0171	*pyrH*	Uridylate kinase	3.3-3.5	3.4	
	B0174	*yaeS*	Undecaprenyl pyrophosphate synthetase	2.1-2.3	2.2	
	B0178	*hlpA*	Periplasmic chaperone	3.1-5.9	4.5	
	B0220	*ykfE*	Hypothetical	1.7-4.6	3.2	1.4
	B0238	*gpt*	Guanine-hypoxanthine phosphoribosyltransferase	1.8-6.4	3.6	2.5
	B0275	*insA_3*	IS1 protein	1.8-2.8	2.4	0.5
	B0360	*yi21_1*	IS2 hypothetical	1.8-4.3	2.9	1.3
	B0390	*aroM*	Aromatic amino acid biosynthesis	1.1-2.3	1.8	0.6
	B0438	*clpX*	Serine protease chaperone	1.8-4.1	3.2	1.3
	B0441	*ppiD*	Peptidyl-prolyl cis-trans isomerase	1.8-2.8	2.2	0.5
	B0452	*tesB*	Acyl-CoA thioesterase II	2.1-4.9	3.5	
	B0474	*adk*	Adenylate kinase	2.9-5.0	4.0	
	B0553	*nmpC*	Outer membrane porin	2.0-3.5	2.6	0.8
	B0578	*nfnB*	Oxygen-insensitive NAD(P)H nitroreductase	2.2-3.0	2.6	
	B0605	*ahpC*	Alkyl hydroperoxide reductase	1.4-2.5	2.1	0.5
	B0623	*cspE*	Cold shock protein	5.3-6.1	5.7	
	B0641	*rlpB*	Minor lipoprotein	1.5-3.0	2.3	0.7
	B0642	*leuS*	Leucine tRNA synthetase	2.3-4.0	3.3	0.9
	B0658	*ybeX*	Putative Mg-Co transport protein	2.8-4.4	3.6	
	B0680	*glnS*	Glutamine tRNA synthetase	3.2-5.0	4.1	
	B0687	*seqA*	Negative regulator of replication initiation	1.6-3.3	2.6	0.9
	B0697	*kdpB*	Potassium-transporting ATPase	1.0-2.7	2.0	0.8
	B0733	*cydA*	} Cytochrome D oxidase subunits	5.3-4.8	5.6	
	B0734	*cydB*		2.3-7.2	4.8	2.4
	B0735	*ybgE*	Hypothetical	1.1-2.7	2.0	0.9
	B0741	*pal*	Peptidoglycan-associated lipoprotein	2.3-3.5	2.9	
	B0756	*galM*	Galactose-1 epimerase	1.8-4.0	2.8	1.1
	B0819	*ybiS*	Putative periplasmic protein	2.4-3.4	2.9	
	B0863	*artI*	Arginine periplasmic binding protein	1.2-2.5	1.9	0.6
	B0884	*infA*	Initiation factor	2.7-6.7	5.3	2.2
	B0889	*lrp*	Leucine-responsive regulatory protein	1.1-4.0	2.4	1.5
	B0907	*serC*	3-phosphoserine aminotransferase	1.4-4.6	3.2	1.6
	B0911	*rpsA*	Ribosomal protein	10.5-22.5	15.5	6.2
	B0929	*ompF*	Outer membrane protein	2.2-5.8	4.1	1.8
	B0987	*ymcD*	} Hypothetical	2.4-3.0	2.7	
	B1003	*yccJ*		2.2-7.3	5.5	2.9
	B1004	*wrbA*	Trp repressor binding protein	1.3-5.1	3.8	2.1
	B1063	*yceB*	Hypothetical	1.6-2.6	2.2	0.5
	B1064	*grxB*	Glutaredoxin	1.3-4.7	2.8	1.8
	B1089	*rpmF*	Ribosomal protein	10.6-13.7	12.2	
	B1093	*fabG*	Fatty acid biosynthesis	5.8-14.5	10.9	4.5
	B1094	*acpP*	Acyl carrier protein	4.0-5.9	4.7	1.0
	B1101	*ptsG*	PTS EII^{glc}	6.8-7.3	7.0	
	B1105	*ycfM*	Hypothetical	1.1-2.1	1.8	0.6
	B1207	*prsA*	Ribose-phosphate pyrophosphokinase	4.7-4.7	4.7	
	B1221	*narL*	} Nitrate reductase	2.3-3.6	3.0	
	B1224	*narG*		6.3-20.1	13.2	
	B1225	*narH*		2.6-22.3	15.6	11.3
	B1226	*narJ*		7.8-20.2	14.9	6.3
	B1237	*hns*	Nucleoid associated protein	2.7-9.0	6.2	3.2
	B1333	*ydaA*	Hypothetical	2.3-2.5	2.4	
	B1376	*ynaF*	Putative filament protein	1.1-5.4	3.6	2.2
	B1403	*yi21_2*	IS hypothetical	1.2-4.2	2.5	1.6
	B1539	*ydfG*	Putative oxidoreductase	1.3-3.3	2.4	1.0
	B1601	*b1601*	Putative transport protein	3.4-3.6	3.5	

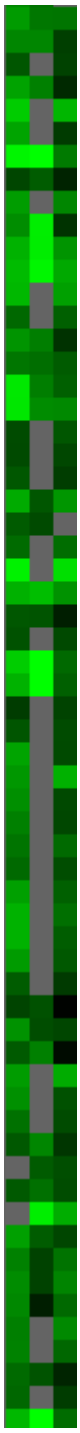
Appendix E.2

Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression in Wild Type						
	B1604	*b1604*	Hypothetical	2.1-5.1	3.6	
	B1641	*slyB*	Outer membrane protein	3.1-5.4	4.2	
	B1642	*slyA*	Transcriptional regulator	2.5-3.9	3.2	
	B1676	*pykF*	Pyruvate kinase	3.0-8.4	6.5	3.0
	B1677	*lpp*	Murein lipoprotein	1.3-4.0	2.6	1.3
	B1712	*himA*	Integration host factor	2.4-4.8	3.6	
	B1713	*pheT*	Phenylalanine tRNA synthetase	2.2-3.0	2.6	
	B1718	*infC*	Initiation factor	5.9-9.5	7.7	
	B1719	*thrS*	Threonine tRNA synthetase	1.8-3.2	2.5	0.7
	B1817	*manX*	} PTS EII^{mannose}	1.3-2.8	2.2	0.8
	B1819	*manZ*		1.1-2.2	1.8	0.6
	B1823	*cspC*	Cold shock protein	4.5-12.7	8.4	4.1
	B1824	*b1824*	Putative cytoplasmic protein	8.2-15.7	11.9	
	B1836	*b1836*	Hypothetical	5.8-5.9	5.8	
	B1852	*zwf*	Glucose-6-phosphate dehydrogenase	3.1-5.4	4.2	
	B1854	*pykA*	Pyruvate kinase	3.3-9.8	6.8	3.3
	B1865	*ntpA*	dATP pyrophosphohydrolase	2.3-2.7	2.5	
	B1902	*yecI*	Ferritin-like protein	2.8-4.7	3.8	
	B1906	*yecH*	} Hypothetical	4.1-5.5	4.8	
	B1983	*b1983*		2.7-3.2	2.9	
	B1997	*yi21_3*	IS hypothetical	1.2-4.2	2.5	1.5
	B2007	*yeeX*	Putative α-helix protein	3.1-4.2	3.8	0.6
	B2113	*mrp*	Putative ATPase	2.1-4.4	3.3	
	B2153	*folE*	GTP cyclohydrolase I	2.6-5.0	3.8	
	B2215	*ompC*	Outer membrane protein	3.8-14.6	10.0	5.6
	B2266	*elaB*	Hypothetical	1.9-5.2	3.7	1.7
	B2285	*nuoE*	NADH dehydrogenase	2.2-3.0	2.6	
	B2293	*yfbT*	Putative phosphatase	2.4-2.5	2.5	
	B2294	*yfbU*	Hypothetical	2.7-3.3	3.0	
	B2296	*ackA*	Acetate kinase	16.0-25.5	19.9	5.0
	B2329	*aroC*	Chorismate synthase	1.7-2.6	2.1	0.5
	B2343	*b2343*	Hypothetical	2.3-3.6	3.0	
	B2416	*ptsI*	PTS EIII^{glc}	8.1-9.3	8.7	
	B2417	*crr*	PTS EIIA^{glc}	3.1-8.9	6.0	
	B2431	*yfeX*	Hypothetical	4.7-4.7	4.7	
	B2465	*tktB*	Transketolase	1.7-2.7	2.2	0.5
	B2476	*purC*	Purine biosynthesis	1.9-7.8	4.6	3.0
	B2477	*nlpB*	Lipoprotein	1.5-5.5	3.6	2.0
	B2478	*dapA*	Dihydropicolinate synthase	1.5-3.7	2.5	1.1
	B2479	*gcvR*	Glycine cleavage system regulator	3.1-4.0	3.6	
	B2499	*purM*	Purine biosynthesis	7.7-7.9	7.8	
	B2501	*ppk*	Polyphosphate kinase	1.8-3.0	2.5	0.6
	B2507	*guaA*	GMP synthetase	2.3-9.0	5.6	
	B2515	*gcpE*	} Hypothetical	4.4-6.6	5.5	
	B2516	*yfgA*		2.4-2.5	2.5	
	B2517	*yfgB*		4.6-4.8	4.7	
	B2519	*pbpC*	Peptidoglycan enzyme	4.0-5.0	4.6	0.6
	B2551	*glyA*	Serine hydroxymethyltransferase	4.3-7.5	5.9	
	B2572	*rseA*	Negative regulator of σ^E	2.5-3.8	3.1	
	B2579	*yfiD*	Putative formate acetyltransferase	2.3-2.8	2.5	
	B2581	*yfiF*	Hypothetical	1.9-3.1	2.4	0.6
	B2595	*yfiO*	Hypothetical lipoprotein	1.1-4.1	3.1	1.7
	B2606	*rplS*	Ribosomal protein	10.3-23.7	17.0	
	B2607	*trmD*	tRNA methyltransferase	8.2-24.5	16.3	
	B2608	*yfjA*	rRNA processing protein	9.1-32.1	18.1	12.3
	B2687	*ygaG*	Autoinducer-2 synthesis	1.9-5.0	3.6	1.6
	B2696	*csrA*	Carbon storage regulator	2.3-5.0	3.7	1.4
	B2697	*alaS*	Alanyl-tRNA synthetase	1.5-3.8	2.6	1.2
	B2699	*recA*	ATP-dependent recombinase	2.4-2.6	2.5	
	B2729	*hypD*	Hydrogenase formation	2.3-2.3	2.3	
	B2779	*eno*	Enolase	2.2-8.4	6.1	3.4
	B2813	*mltA*	Murein transglycosylase	2.0-2.2	2.1	

Appendix E.3

Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression in Wild Type						
	B2860	*yi22_4*	} IS2 hypothetical	4.5-6.2	5.3	
	B2861	*yi21_4*		2.4-3.7	2.9	0.7
	B2891	*prfB*	Release factor	2.8-4.3	3.5	
	B2894	*xerD*	Site-specific recombinase	2.9-3.5	3.2	
	B2913	*serA*	Phosphoglycerate dehydrogenase	3.8-3.9	3.8	
	B2925	*fba*	Fructose-bisphosphate aldolase	2.2-5.1	3.5	1.4
	B2926	*pgk*	Phosphoglycerate kinase	1.6-4.2	3.2	1.4
	B2942	*metK*	Methionine metabolism	2.1-3.6	2.9	
	B2959	*yggL*	Hypothetical	1.2-4.0	2.7	1.4
	B3035	*tolC*	Outer membrane channel	1.5-3.6	2.7	1.1
	B3071	*yqjI*	} Hypothetical	2.7-3.9	3.3	
	B3097	*yqjC*		1.4-5.5	3.2	2.1
	B3099	*yqjE*		1.1-2.8	2.2	1.0
	B3157	*yhbT*		2.7-3.4	2.9	0.4
	B3163	*nlpI*	Putative lipoprotein precursor	1.9-5.7	4.1	2.0
	B3165	*rpsO*	Ribosomal protein	4.9-10.1	7.4	2.6
	B3175	*secG*	Secretion protein	4.1-7.8	6.0	
	B3179	*ftsJ*	Cell division protein	2.2-2.8	2.5	
	B3185	*rpmA*	} Ribosomal proteins	4.3-5.6	5.0	
	B3186	*rplU*		5.4-17.7	12.1	6.2
	B3230	*rpsI*		2.3-4.1	3.2	
	B3231	*rplM*		29.5-60.1	42.9	15.7
	B3256	*accC*	Acetyl CoA carboxylase	6.4-10.1	8.3	
	B3260	*yhdG*	Putative dehydrogenase	11.0-12.8	11.9	
	B3280	*yrdB*	} Hypothetical	2.1-2.2	2.1	
	B3284	*smg*		1.1-2.7	2.0	0.9
	B3285	*smf_2*		2.5-4.7	3.7	1.1
	B3287	*def*	Peptide deformylase	2.9-4.5	3.7	
	B3295	*rpoA*	RNA polymerase α subunit	13.9-40.3	23.0	15.0
	B3296	*rpsD*	} Ribosomal proteins	9.0-31.6	20.3	
	B3297	*rpsK*		19.4-32.9	26.1	6.8
	B3299	*rpmJ*		15.8-22.5	19.6	3.5
	B3300	*prlA*	SecY secretion protein	7.6-28.2	15.7	11.0
	B3301	*rplO*	} Ribosomal proteins	28.3-28.6	28.5	
	B3304	*rplR*		11.8-32.6	20.7	10.7
	B3305	*rplF*		13.8-21.3	17.5	
	B3307	*rpsN*		21.5-28.5	25.0	
	B3308	*rplE*		9.2-10.2	9.8	
	B3310	*rplN*		14.4-17.1	15.7	
	B3313	*rplP*		6.3-8.6	7.4	
	B3315	*rplV*		6.8-31.8	19.3	
	B3316	*rpsS*		2.7-26.7	10.9	13.7
	B3317	*rplB*		25.3-30.9	28.1	
	B3318	*rplW*		26.2-44.8	35.5	
	B3320	*rplC*		7.5-35.1	20.5	13.9
	B3339	*tufA*	Peptide elongation factor	7.5-20.9	16.4	7.7
	B3340	*fusA*	Elongation factor	13.6-14.4	14.0	
	B3342	*rpsL*	Ribosomal protein	17.4-27.2	22.3	
	B3400	*hslR*	Heat shock protein	1.5-2.2	2.0	0.4
	B3431	*glgX*	Glycosyl hydrolase	1.2-3.3	2.2	1.1
	B3458	*livK*	Leucine transport protein	2.1-2.5	2.3	
	B3619	*rfaD*	LPS biosynthesis	1.2-3.7	2.7	1.3
	B3637	*rpmB*	Ribosomal protein	7.9-10.6	9.3	
	B3649	*rpoZ*	RNA polymerase ω subunit	1.9-3.1	2.5	0.6
	B3687	*ibpA*	Heat shock protein	3.9-7.1	5.5	
	B3732	*atpD*	} F_1F_0ATPase	1.9-5.7	4.3	2.1
	B3733	*atpG*		3.8-6.9	5.4	
	B3734	*atpA*		7.7-11.2	9.4	
	B3735	*atpH*		9.5-13.2	11.4	
	B3736	*atpF*		6.5-11.3	8.9	
	B3781	*trxA*	Thioredoxin	2.9-4.1	3.5	
	B3782	*rhoL*	Rho operon leader peptide	5.1-8.9	7.0	
	B3783	*rho*	Transcription termination factor Rho	4.0-5.0	4.5	

Appendix E.4

Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression in Wild Type						
	B3836	*tatA*	} Twin-arginine translocation proteins	4.1-6.5	5.0	1.4
	B3838	*tatB*		2.2-5.1	4.1	1.7
	B3860	*dsbA*	Disulfide bond formation	2.2-2.9	2.5	
	B3916	*pfkA*	Phosphofructokinase	1.7-7.2	4.5	2.8
	B3936	*rpmE*	Ribosomal protein	9.1-11.8	10.5	
	B3945	*gldA*	Glycerol dehydrogenase	2.0-7.3	4.7	
	B3980	*tufB*	Elongation factor	4.4-22.4	15.4	9.7
	B3981	*secE*	Protein translocation	1.5-3.1	2.3	0.8
	B3982	*nusG*	Transcription antitermination factor	5.1-6.7	5.9	
	B3984	*rplA*	} Hypothetical	1.9-17.8	8.4	8.3
	B3985	*rplJ*		6.0-17.6	10.7	6.1
	B3986	*rplL*		7.8-19.7	12.4	6.4
	B3987	*rpoB*	RNA polymerase β subunit	6.9-9.3	8.1	
	B3988	*rpoC*	RNA polymerase β' subunit	3.2-3.6	3.4	
	B4000	*hupA*	Histone-like DNA binding protein	1.9-6.0	4.1	2.1
	B4025	*pgi*	Glucosephosphate isomerase	3.0-4.2	3.7	0.6
	B4142	*mopB*	} GroEL chaperonin	3.2-16.6	8.1	7.4
	B4143	*mopA*		5.3-16.3	9.0	6.3
	B4152	*frdC*	Fumarate reductase	2.5-2.9	2.7	
	B4167	*yjeF*	Hypothetical	2.4-2.5	2.4	
	B4171	*miaA*	tRNA delta-2-isopentenylpyrophosphate transferase	2.1-2.9	2.5	
	B4172	*hfq*	RNA chaperone	2.9-7.9	5.7	2.6
	B4173	*hflX*	GTP binding protein	2.4-3.0	2.7	
	B4177	*purA*	Purine biosynthesis	3.7-3.8	3.7	
	B4202	*rpsR*	Ribosomal protein	14.8-18.7	16.7	
	B4226	*ppa*	Inorganic pyrophosphatase	5.9-10.0	8.2	2.1
	B4234	*yjgA*	Putative α-helix protein	1.5-2.9	2.4	0.8
	B4255	*yjgD*	Hypothetical	2.4-2.7	2.6	
	CSRB	*csrB*	} sRNA regulators of CsrA	3.6-28.1	14.6	12.4
	CSRC	*csrC*		3.2-32.6	14.8	15.7
	ECS2333	*blr*	β-lactam resistance protein	2.1-2.2	2.2	
	S0013	*mkaD*	Virulence plasmid associated gene	2.5-2.8	2.7	
	S0088	*ospD3*	TTSS secreted effector	2.5-7.3	4.9	
	S0131	*virB*	Transcriptional activator of TTSS	6.2-8.6	7.4	
	S0137	*ipgD*	} TTSS proteins	2.4-5.7	4.0	
	S0140	*icsB*		2.4-4.7	3.5	
	S0150	*mxiL*		3.6-4.1	3.9	
	S0154	*mxiD*		2.5-3.6	3.1	
	S0157	*spa15*		3.2-7.0	5.1	
	S0160	*spa32*		3.8-8.7	6.2	
	S0161	*spa33*		3.1-8.6	5.9	
	S0166	*spa-orf10*		2.9-6.5	4.7	
	S0179	*sp0179*		2.0-3.1	2.5	
	SF0373	*s0373*	Hypothetical	1.0-2.7	2.0	0.9
	SF0391	*hupB*	DNA binding protein	2.3-5.9	3.6	2.0
	SF0676	*tatE*	Twin-arginine translocation protein	1.2-4.7	3.0	1.8
	SF1323	*hns*	Nucleoid associated protein	6.6-8.0	7.3	
	SF1800	*slyB*	Putative outer membrane protein	2.7-5.8	4.2	
	SF2222	*rfbE*	LPS biosynthesis	2.5-3.9	3.2	
	SF3951	*atpE*	F_1F_0 ATPase subunit	1.9-5.1	3.3	1.6
	SRAJ	*sraJ*	} sRNA	2.6-3.0	2.8	
	SSRS	*ssrS*		2.5-3.9	3.1	0.7
	T44	*t44*		8.1-17.8	13.0	
	Z1134	*z1134*	IS3 putative transposase	2.3-6.9	4.1	2.5
	Z1150	*z1150*	} IS hypothetical	2.2-4.8	3.7	1.3
	Z1589	*z1589*		2.2-5.4	4.2	1.7
	Z3095	*z3095*	Putative prophage transposase	1.4-5.2	3.4	1.9
	Z3162	*z3162*	IS transposase	4.7-5.3	5.0	
	Z4229	*prfB*	Release factor	3.5-4.9	4.2	
	Z4695	*z4695*	Putative bacterioferritin	1.5-3.6	2.6	1.0
	Z5325	*z5325*	Putative outer membrane lipoprotein	2.1-3.5	2.8	
	Z5401	*z5401*	Putative cytoplasmic protein	3.7-30.8	14.6	14.3

Appendix E.5

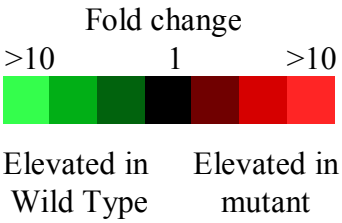
Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression in *arcA fnr*						
	B0114	*aceE*	Pyruvate dehydrogenase	1.8-3.9	2.8	1.1
	B0116	*lpdA*	Lipoamide dehydrogenase (NADH)	2.8-3.4	3.0	0.4
	B0306	*ykgE*	Putative dehydrogenase	2.1-2.6	2.3	
	B0565	*ompT*	Outer membrane protein	1.9-2.3	2.1	0.2
	B0674	*asnB*	Asparagine synthetase	2.8-5.5	4.1	1.3
	B0681	*ybfM*	Putative outer membrane protein	2.1-2.7	2.4	
	B0720	*gltA*	Citrate synthase	5.6-16.4	9.6	5.9
	B0721	*sdhC*	Succinate dehydrogenase	4.8-22.9	11.2	10.1
	B0722	*sdhD*		4.6-17.2	12.8	7.1
	B0723	*sdhA*		8.8-17.8	11.8	5.2
	B0724	*sdhB*		1.9-5.5	3.6	1.8
	B0725	*b0725*	Hypothetical	2.5-11.3	6.3	4.5
	B0726	*sucA*	Succinyl transferase	4.8-6.5	5.5	0.9
	B0727	*sucB*		3.9-7.5	6.0	1.9
	B0728	*sucC*		2.3-7.2	5.1	2.5
	B0729	*sucD*		4.6-9.5	6.4	2.7
	B0751	*pnuC*	Nucleoside transporter	2.2-2.2	2.2	
	B0811	*glnH*	Glutamine transport	2.5-4.0	3.5	0.8
	B0960	*yccS*	Putative membrane protein	1.3-2.3	1.9	0.5
	B0990	*cspG*	Cold shick protein	2.1-2.3	2.2	
	B1136	*icdA*	Isocitrate dehydrogenase	2.3-6.0	3.9	1.9
	B1137	*ymfD*	Hypothetical	2.8-3.7	3.3	
	B1145	*b1145*	Putative phage repressor	2.3-2.7	2.5	
	B1157	*b1157*	Putative tail fiber protein	2.2-2.3	2.2	
	B1268	*yciQ*	Putative membrane protein	1.7-2.6	2.2	0.5
	B1276	*acnA*	Aconitase A	1.6-9.3	4.6	4.1
	B1311	*ycjO*	Putative transport permeease	2.1-2.3	2.2	
	B1324	*tpx*	Thiol peroxidase	1.8-4.5	3.0	1.4
	B1397	*b1397*	Putative acyl transferase	2.2-2.5	2.3	
	B1422	*b1422*	Hypothetical transcriptional regulator	2.5-3.5	3.0	
	B1454	*b1454*	Putative transferase	1.2-2.7	2.1	0.8
	B1501	*yedP*	Putative oxidoreductase	2.1-2.4	2.2	
	B1582	*ynfA*	Putative inner membrane protein	1.4-2.8	2.1	0.7
	B1622	*malY*	Maltose regulon modulator	2.1-2.3	2.2	
	B1648	*ydhL*	Hypothetical	1.4-4.3	2.8	1.4
	B1780	*yeaD*		2.4-3.6	3.0	
	B1853	*yebK*	Putative hex regulon repressor	4.0-4.2	4.1	
	B1916	*sdiA*	Cell division regulatory protein	2.2-2.2	2.2	
	B1943	*fliK*	Flagellar synthesis	2.1-2.2	2.1	
	B1999	*yeeP*	Hypothetical	2.1-2.7	2.4	
	B2091	*gatD*	Galactitol-1-phosphate dehydrogenase	2.2-3.1	2.6	
	B2115	*molR_1*	Molybdate metabolism regulator	1.4-3.2	2.3	0.9
	B2150	*mglB*	D-galactose periplasmic binding protein	1.2-5.0	3.1	1.9
	B2167	*fruA*	PTS EIIBC^{fructose}	2.3-2.3	2.3	
	B2452	*eutH*	Ethanolamine utilization	1.4-2.1	1.9	0.4
	B2555	*yfhG*	Putative α-helix protein	2.3-2.8	2.6	
	B2557	*purL*	Purine biosynthesis	2.6-3.2	2.9	
	B2720	*hycF*	Formate hydrogen lyase subunit	2.4-2.5	2.5	
	B2902	*ygfF*	Putative oxidoreductase	2..1-2.3	2.2	
	B2979	*glcD*	Glycolate oxidase subunit	2.8-7.1	4.9	
	B3082	*ygjM*	Hypothetical	2.1-2.3	2.2	
	B3141	*agaI*	Putative galactosamine-6-phosphate isomerase	1.8-2.0	1.9	0.1
	B3160	*yhbW*	Possible mono-oxygenase	2.0-2.2	2.1	
	B3236	*mdh*	Malate dehydrogenase	1.9-4.5	2.8	1.5
	B3477	*nikB*	Nickel transport peremease	2.4-2.7	2.5	
	B3505	*trs5_11*	IS5 transposase	1.8-3.0	2.3	0.6
	B3521	*yhjC*	Putative transcriptional regulator	1.4-2.3	1.9	0.4
	B3532	*yhjN*	Hypothetical	1.2-2.6	2.0	0.8
	B3603	*lldP*	Lactate permease	12.4-26.8	21.7	8.1
	B3604	*lldR*		4.0-18.6	10.1	7.6
	B3605	*lldD*		9.7-14.8	12.9	2.8

Appendix E.6

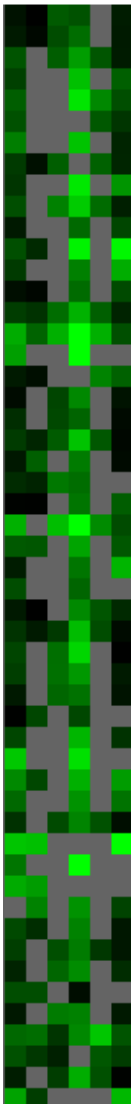
Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression in *arcA fnr*						
	B3744	*asnA*	Asparagine synthetase	4.3-7.8	6.2	1.8
	B3952	*pflC*	Pyruvate formate lyase activating enzyme	1.8-2.2	2.0	0.2
	B4032	*malG*	Maltose permease	1.3-2.8	2.1	0.8
	B4083	*yjcS*	Putative hydrolase	2.1-2.4	2.3	
	B4091	*phnQ*	} Hypothetical	1.8-2.8	2.2	0.5
	B4188	*yjfN*		2.5-2.8	2.6	
	B4189	*yjfO*	Putative lipoprotein	3.5-3.6	3.5	
	B4193	*sgaT*	} L-ascorbate utilization	2.8-3.2	3.0	
	B4194	*sgaB*		2.2-2.4	2.3	
	B4196	*sgaH*		2.7-3.1	2.9	
	B4302	*sgcA*	Putative PTS EII	2.3-2.8	2.6	
	B4376	*osmY*	Hyperosmotic stress response protein	2.4-3.9	3.3	0.7
	B4402	*yjjY*	Hypothetical	1.7-3.0	2.4	0.6
	C0067	*imp*	Organic solvent tolerance	2.3-2.5	2.4	
	C0719	*c0719*	Hypothetical	1.9-2.7	2.2	0.4
	ECS1988	*ecs1988*	} Hypothetical	2.5-4.2	3.4	
	ECS2980	*ninE*		1.5-2.9	2.2	0.7
	ECS2989	*ecs2989*	Putative regulatory protein	2.1-2.2	2.2	
	SF0839	*ybjH*	} Hypothetical	1.5-4.9	2.9	1.7
	SF3185	*s3185*		1.8-3.3	2.5	0.7
	Z0024	*z0024*	Putative fimbrial protein	2.0-2.6	2.3	0.3
	Z0255	*z0255*	Putative membrane protein	2.1-2.3	2.2	0.1
	Z0337	*z0337*	} Putative regulator	2.0-2.1	2.1	
	Z0346	*z0346*		1.8-2.5	2.2	0.3
	Z0418	*z0418*	Putative permease	2.1-3.3	2.8	0.6
	Z0872	*ybgD*	Putative fimbrial protein	2.6-2.8	2.7	
	Z0895	*z0895*	Putative glutamate mutase	2.2-8.1	4.3	3.2
	Z0963	*z0963*	Unknown prophage protein	2.2-4.1	3.2	
	Z0979	*z0979*	Putative prophage tail component	2.1-2.1	2.1	
	Z1177	*terF*	Tellurium/colicin resistance, phage inhibition	2.2-3.5	2.8	
	Z1183	*z1183*	} Hypothetical	2.5-3.8	3.2	
	Z1225	*z1225*		2.3-4.3	3.3	
	Z1486	*z1486*		2.0-3.1	2.4	0.5
	Z1491	*z1491*		2.1-2.1	2.1	
	Z1576	*z1576*		2.0-2.3	2.1	0.2
	Z1781	*z1781*		2.6-2.9	2.7	
	Z1932	*z1932*		1.9-2.3	2.1	0.2
	Z2068	*z2068*		2.3.-3.3	2.8	
	Z2148	*z2148*		2.0-3.3	2.5	0.7
	Z2359	*z2359*	Putative prophage capsid protein	1.1-2.1	1.8	0.6
	Z2560	*z2560*	} Hypothetical	2.1-2.4	2.3	
	Z2984	*z2984*		2.1-3.0	2.5	0.5
	Z3086	*z3086*	Putative minor tail protein	1.4-37.8	13.8	20.8
	Z3202	*wbdO*	Glycosyl transferase	2.7-2.9	2.8	
	Z3306	*z3306*	Hypothetical	2.1-2.2	2.2	
	Z3307	*z3307*	Putative phage tail fiber protein	1.8-2.5	2.2	0.3
	Z3316	*z3316*	Unknown prophage protein	1.4-2.9	2.1	0.7
	Z3349	*z3349*	Putative DNA methyl transferase	2.4-4.2	3.3	
	Z3598	*z3598*	Putative fimbrial protein	2.6-3.3	3.0	
	Z3921	*z3921*	Hypothetical	2.5-6.2	4.4	
	Z5109	*escD*	Type III secretion system protein	2.1-2.5	2.3	
	Z5129	*z5129*	Hypothetical	2.2-3.3	2.8	
	Z5135	*escR*	Type III secretion system protein	1.8-2.4	2.1	0.3
	Z5522	*z5522*	Putative citrate permease	1.7-3.3	2.7	0.8
	Z5618	*z5618*	Putative sorbitol-6-phosphate 2-dehydrogenase	2.0-2.1	2.1	0.1
	Z5902	*z5902*	Putative helicase	1.6-2.7	2.2	0.5
	Z6025	*z6025*	} Hypothetical prophage proteins	2.2-2.9	2.6	
	Z6046	*z6046*		1.4-4.4	2.9	1.5
	Z6080	*exoP*	Putative exodeoxyribonuclease	3.1-4.2	3.6	

Appendix F: Microarrays demonstrate transcriptional changes between *S. flexneri* wild type and the *arcA fnr* mutant when grown anaerobically in iron-deplete media

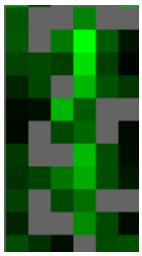
Bacteria were grown aerobically without added iron to early logarithmic phase in a chemostat and harvested 15 minutes after oxygen depletion. RNA was then isolated, reverse transcribed, and labeled for use in microarray hybridizations. Average fold changes are derived from at least two experiments. Green spots indicate genes elevated anaerobically in wild type, while red spots indicate greater anaerobic expression in the *arcA fnr* mutant. Grey spots did not pass the spot filters. * indicates that standard deviations were only included for genes that met the quality control in at least three experiments.



Appendix F.1

Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression in Wild Type						
	B0054	*imp*	Organic solvent tolerance	1.0-2.5	1.7	0.7
	B0082	*yabC*	Putative apolipoprotein	1.1-3.0	1.8	0.8
	B0435	*bolA*	Putative murein regulator	1.3-4.8	2.7	1.3
	B0733	*cydA*	Cytochrome D terminal oxidase subunit	1.9-6.9	3.8	2.7
	B0903	*pflB*	Formate acyltransferase	2.2-9.9	4.6	3.6
	B0966	*yccV*	} Hypothetical	1.7-2.4	2.1	0.4
	B1003	*yccJ*		1.4-7.1	4.0	2.9
	B1004	*wrbA*	Trp repressor binding protein	1.2-2.6	1.9	0.7
	B1060	*yceP*	Hypothetical	1.7-10.3	5.5	4.4
	B1064	*grxB*	Glutaredoxin	1.4-7.6	3.6	2.4
	B1127	*pepT*	Putative peptidase	1.3-2.9	2.1	0.8
	B1224	*narG*	} Nitrate reductase	1.5-11.9	6.8	5.7
	B1226	*narJ*		1.8-5.7	3.7	1.9
	B1246	*oppD*	Oligopeptide permeasee	1.1-2.9	1.8	0.9
	B1283	*osmB*	Osmotically induced lipoprotein	1.4-5.9	2.7	1.7
	B1376	*ynaF*	Putative filament protein	2.2-16.9	6.5	5.3
	B1541	*b1541*	Putative cytoplasmic protein	4.9-15.1	10.0	
	B1661	*cfa*	Cyclopropane fatty acid synthase	1.2-3.7	2.2	1.2
	B1681	*sufD*	} Fe-S cluster assembly	1.1-3.9	2.1	1.3
	B1682	*sufC*		1.1-2.7	1.9	0.7
	B1713	*pheT*	Phenylalanine tRNA synthetase	1.1-7.1	2.7	2.2
	B1718	*infC*	Initiation factor	1.1-3.4	2.1	1.1
	B1902	*yecI*	Ferritin-like protein	1.4-3.4	2.3	1.0
	B1906	*yecH*	Hypothetical	1.0-3.2	2.0	1.1
	B2215	*ompC*	Outer membrane protein	2.1-13.2	6.4	4.2
	B2296	*ackA*	Acetate kinase	2.4-5.2	3.1	1.4
	B2726	*hypA*	Hydrogenase maturation	1.3-6.1	3.6	2.4
	B3007	*b3007*	Hypothetical	2.2-2.8	2.5	
	B3024	*ygiW*	Putative outer membrane protein	1.0-3.9	2.0	1.2
	B3045	*yi22_5*	IS2 hypothetical	1.1-6.6	2.4	2.1
	B3110	*yhaO*	Putative transport permease	1.0-8.5	3.7	3.4
	B3207	*yrbL*	Hypothetical	1.3-4.6	2.7	1.5
	B3287	*def*	Peptide deformylase	1.2-3.1	2.1	1.0
	B3349	*slyD*	Peptidyl-prolyl isomerase	1.0-2.1	1.7	0.6
	B3362	*yhfG*	Hypothetical	1.7-6.2	3.3	2.5
	B3365	*nirB*	NADH-dependent nitrite reductase	7.5-9.4	8.5	
	B3408	*feoA*	} Fe^{2+} transport	2.0-5.8	4.2	1.6
	B3409	*feoB*		2.8-4.3	3.4	0.8
	B3448	*yhhA*	Putative outer membrane protein	1.2-3.8	2.2	1.0
	B3510	*hdeA*	Acid responsive protein	6.9-38.9	17.7	18.4
	B3512	*yhiE*	Hypothetical	3.2-18.7	11.0	
	B3517	*gadA*	Glutamate decarboxylase	4.8-5.5	5.2	
	B3612	*yibO*	Putative phosphoglycerate mutase	2.0-4.3	3.3	1.2
	B3729	*glmS*	Glutamine-fructose-6-phosphate transaminase	1.3-3.7	2.2	1.1
	B3919	*tpiA*	Triosephosphate isomerase	1.3-3.5	2.1	0.8
	B3922	*yiiS*	Hypothetical	1.4-4.1	2.5	1.2
	B4401	*arcA*	Anaerobic regulator	1.1-2.2	1.8	0.6
	C0343	*c0343*	sRNA	1.3-3.7	2.4	1.3
	CSRB	*csrB*	} sRNA regulators of CsrA	1.7-7.3	3.5	2.0
	CSRC	*csrC*		1.4-2.4	1.9	0.4
	ECS5328	*ecs5328*	Hypothetical	1.0-5.7	2.4	1.9
	GCVB	*gcvB*	sRNA regulator of glycine cleavage system	1.9-6.3	4.4	2.3

Appendix F.2

Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression in Wild Type						
	S0003	*ospB*	} TTSS secreted effectors	2.3-3.7	3.0	6.5
	S0007	*ospC4*		1.2-16.7	5.2	
	S0016	*sp0016*	Hypothetical receptor kinase	1.1-9.4	3.0	3.1
	S0088	*ospD3*	TTSS secreted effector	1.4-6.2	2.7	2.0
	S0198	*ushA*	UDP-sugar hydrolase	1.1-6.2	2.7	2.4
	S0253	*msbB*	Lipid A acylating protein	1.1-2.8	1.8	0.8
	SF0237	*s0237*	Putative terminase	1.2-6.5	2.9	2.4
	SF1370	*osmB*	Osmotically induced lipoprotein, same as b1283	1.3-5.5	2.6	1.6
	SPF	*spf*	sRNA	1.9-3.0	2.3	0.6
	Z4695	*z4695*	Bacterioferritin	1.1-4.1	2.2	1.3
	Z5401	*z5401*	Putative cytoplasmic protein	1.2-2.2	1.8	0.4

Spot Image	B Number	Gene	Function	Range	Average Fold-change	S.D.*
Increased Anaerobic Expression in *arcA fnr*						
	B0035	*caiE*	Stimulates carnitine racemase activity	1.1-2.9	2.0	1.0
	B0116	*lpdA*	Lipoamide dehydrogenase (NADH)	1.7-14.2	7.7	6.4
	B0126	*yadF*	Putative carbonic anhydrase	1.6-2.1	1.9	0.3
	B0720	*gltA*	Citrate synthase	4.1-31.4	20.3	14.4
	B0725	*b0725*	Hypothetical	9.2-18.6	12.3	5.4
	B0726	*sucA*	} Succinyl transferase	2.4-19.0	10.7	
	B0727	*sucB*		3.0-12.0	5.4	4.4
	B0728	*sucC*		2.4-20.6	11.5	
	B0729	*sucD*		6.8-19.9	13.4	
	B0741	*pal*	Peptidoglycan-associated lipoprotein	1.1-5.0	2.5	1.5
	B0787	*ybhM*	Hypothetical	1.1-2.8	1.9	0.9
	B0811	*glnH*	Periplasmic glutamine-binding protein	4.1-6.3	5.2	
	B0814	*ompX*	Outer membrane protein	1.1-4.9	2.3	1.3
	B0819	*ybiS*	Putative periplasmic protein	2.1-2.7	2.3	0.3
	B0891	*lolA*	Outer membrane lipoprotein carrier	1.4-3.2	2.5	0.9
	B1136	*icdA*	Isocitrate dehydrogenase	1.8-25.5	9.8	13.6
	B1308	*pspE*	Phage shock protein E	2.3-4.0	3.1	
	B1334	*fnr*	Anaerobic regulator	1.1-3.1	2.1	0.8
	B1407	*ydbD*	} Hypothetical	2.0-5.9	4.5	2.1
	B1777	*b1777*		2.6-2.7	2.6	
	B2063	*yegH*	Putative transport protein	1.3-2.4	2.0	0.6
	B3114	*tdcE*	Keto-acid formate acetyltransferase	1.2-2.4	2.0	0.7
	B3236	*mdh*	Malate dehydrogenase	2.3-13.4	5.7	4.6
	B3263	*yhdU*	Hypothetical	1.1-2.3	1.7	0.6
	B3603	*lldP*	Lactate permease	8.7-98.2	43.0	36.5
	B3620	*rfaF*	LPS core biosynthesis	2.3-3.2	2.7	
	B4054	*tyrB*	Tyrosine aminotransferase	2.3-2.9	2.6	
	B4147	*efp*	Elongation factor	2.3-6.7	4.5	
	B4217	*ytfK*	Hypothetical	1.2-3.5	1.9	1.0
	B4300	*sgcR*	Putative regulatory protein	2.2-5.3	3.7	
	B4376	*osmY*	Hyperosmotic stress response protein	1.7-22.0	10.7	8.8
	SF0559	*s0559*	Putative homeobox protein	1.0-3.7	2.5	1.2
	SF4052	*iutA*	} Aerobactin synthesis and transport	1.5-7.5	3.9	2.4
	SF4053	*iucD*		1.1-7.6	3.2	2.4
	SSRA	*ssrA*	sRNA	1.2-4.7	2.6	1.3
	Z1812	*z1812*	Putative prophage chaperone	1.1-2.4	1.7	0.7
	Z5050	*waaD*	Putative LPS biosynthesis enzyme	2.0-2.1	2.0	
	Z5978	*z5978*	Putative inner membrane protein	1.3-7.2	3.4	2.2

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